MOLECULAR CHARACTERIZATION OF THE SMALL MITOCHONDRIAL DNAS OF MAIZE

By

Alan G. Smith

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1985

ACKNOWLEDGEMENTS

I would would like to thank Dr. Daryl Pring for his enthusiasm, complete support, and and for the instruction he has given me as a graduate student. I thank Dr. Prem Chourey for his keen scrutiny of data, and for his guidance. I also thank Drs. L. Curtis Hannah and David Loschke for their review of the dissertation, and their friendly criticism of my research. I appreciate the help of Dr. James McNay for his hands on instructions, creative thinking, and the identification of the dreaded Florida rattle-toad (*Bufo toxicus*). I am grateful to Dr. Chris Chase for her technical assistance, and Dr. Roger Wise and Mr. John Kennell for correcting a plethora of spelling and grammatical errors.

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December, 1985

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Major Department: Plant Pathology

The mitochondrial genome of Black Mexican maize was found to consist of the principal genome, two minicircular mitochondrial DNAs (1913 and 1445 base pairs (bp) in size), and a 2.3 kb linear DNA. The copy number, replication, distribution, expression, and sequence of the two minicircular DNAs were examined. The copy number of the minicircles varied between cell suspension and the plant and among the growth phases of the cell suspension. The minicircular DNAs were used as models of mitochondrial DNA replicons because they can be separated from the complex principal genome.

Incorporation of radioactive label to a high specific activity occurred in logarithmic cultures whereas no incorporation was detected in stationary phase cultures. When stationary phase cultures renewed DNA replication, there was a temporal synthesis of DNA as well as a preferential incorporation of radioactive label into the 1.9 kb minicircle and proplastid DNA. This could reflect a need for more

proplastids and 1.9 kb minicircles or a differences in control of DNA synthesis.

The distribution of the two circular DNAs among cytoplasms of maize is variable. There was no homology between either minicircle and any other maize mitochondrial DNA. In a survey of maize cytoplasms neither the 1.9 nor 1.4 kb minicircles were present in all cytoplasms examined. No cytoplasm tested carried the 1.4 kb minicircle without the 1.9 kb minicircle. Those cytoplasms which did not carry either DNA were examined for sequences homologous to the 1.9 and 1.4 kb minicircles in the nucleus. One of the lines examined had nuclear sequences homologous to the 1.9 and 1.4 kb minicircles while other lines were lacking any homology to the DNAs.

Transcripts from the 1.9 and 1.4 kb minicircles were detected in Black Mexican cell suspension cultures. The longest open reading frame of the 1.4 kb minicircle is 231 bp long and it corresponds to the transcribed sequence. The 1.4 kb minicircle contains 62 bp of near perfect homology to the 1.9 kb minicircle and direct repeats of 16 and 13 bp in length. Two imperfect repeats of 12 bp are located in a high A+T region which may serve as an origin of replication for the minicircle.

INTRODUCTION

Maize mitochondrial DNA is characterized by classes of small minicircular and minilinear DNAs in addition to high molecular weight chromosomal DNA. Depending upon the cytoplasm examined, the minilinear and minicircular mtDNAs include various combinations of linear DNA molecules ranging in size from 7.4 to 2.1 kb and circular DNA molecules ranging in size from 1.9 to 1.4 kb (Pring and Lonsdale 1985). Minilinear and minicircular DNAs have been described in many plant species; however, their function and interaction with principal genome of the mitochondrial DNA is not well understood.

Replication of the higher plant mitochondrial genome is essential to its inheritance in both somatic and sexual generations. The understanding of mitochondrial DNA replication in higher plants has not progressed as rapidly as that of the yeast and mammalian mitochondria, primarily because of the large size and heterogeneity of the plant mitochondrial genome. Physical maps of the mitochondrial genome of three plant species have been completed and the mapping of structural genes is still incomplete. Small DNA molecules, which are found in mitochondria of many species, have been identified in the maize mitochondria (Levings et al. 1979; Kemble and Bedbrook 1980).

These small circular molecules can be used as models of DNA synthesis in the maize mitochondrion. The existence of two distinct minicircular molecules within the mitochondrion made the system

analogous to the many circular DNAs of the principal genome. These DNAs are stably inherited over many generations. This stability is thought to be conferred by functioning origins of replication on the molecules.

This study was initiated to examine the molecular characteristics of two minicircular DNAs found within the maize mitochondrion. Replication, distribution, transcription and nucleotide sequence were studied. These small DNAs were considered model for the much larger and more complex principal genome of the maize mitochondrion. It is not possible to isolate the individual circular molecules of the principal genome as discrete size classes, however portions of the molecules may be cloned by recombinant DNA technology. The minicircular DNAs can be isolated as discrete molecules from the principal genome and provide a means to follow individual classes of circular DNAs.

Synthesis of the minicircular DNAs was followed in a suspension culture of maize. The cell suspension culture is useful because of the rapid cell divisions. This necessitates that the mitochondrial genome be replicated as the cells divide to produce increased numbers of mitochondria for the daughter cells. It would be expected that there is much more mitochondrial DNA synthesis occurring in this type of undifferentiated tissue, relative to that of the terminally-differentiated somatic cells of the plant.

In order to better understand the minicircular DNAs as mitochondrial replicons, some of the basic features of these molecules were determined. This included the distribution of the minicircular DNA sequences within the cell and among the other maize cytoplasms. The relationship of these DNAs to each other and with other DNAs found within the maize mitochondrion was determined. From these data it was

possible to hypothesize on the origin of these DNAs. Transcription of the minicircles was assayed to determine if a function or phenotype could be ascribed to these molecules. The complete nucleotide sequence of the 1.4 kb minicircle was determined and compared with other mitochondrial sequences.

LITERATURE REVIEW

Mitochondrial Genome Function and Structure

Mitochondrial Function

The mitochondrion provides a compartment within the cell for the reactions of the electron-transport system, oxidative phosphorylation, fatty acid oxidation, and the citric acid cycle (Tzagoloff 1982). These reactions are essential for an obligately aerobic organism, providing it with reducing power for the production of ATP. The mitochondrial DNA encodes for mRNAs, rRNAs and tRNAs which are used to synthesize mitochondrial encoded proteins. The mitochondrial genome is apparently associated with several plant phenotypes. In maize [*Zea mays* L.] these include cytoplasmic male sterility, susceptibility to the plant pathogens *Cochliobolus heterostrophus*, and *Phyllosticta maydis*, and susceptibility to an insecticide (for reviews see Laughnan and Gabay-Laughnan 1983; Levings and Pring 1979; Duvick 1965; Ullstrup 1972).

The evidence for the involvement of the mitochondrion in these phenotypes is convincing although not definitive. These phenotypes are maternally inherited in maize (Levings and Pring 1976; Pring and Levings 1978). The cytoplasms of maize are classified into fertile (N) and male sterile (C, S, and T). The sterile cytoplasms which are grouped by nuclear genes which restore male fertility, can also be separated by

their mitochondrial DNA restriction endonuclease pattern. The chloroplast DNA restriction endonuclease patterns of N, T_1 and C cytoplasms are indistinguishable, the chloroplast DNA of S cytoplasm is unique [Pring and Levings 1978]. The cytoplasms of maize can also be distinguished by the polypeptides synthesized in vitro by isolated mitochondria (Forde, Oliver and Leaver 1978; Forde and Leaver 1980; Forde et al. 1980).

Cochliobolus heterostrophus race T causes disease only on the T cytoplasms of maize (Hooker et al. 1970). The earliest cytological effect of the pathotoxin produced by the fungus is on the mitochondrion of T cytoplasm (Aldrich et al. 1977). Tissue culture of T cytoplasm maize with and without selection with the pathotoxin, was used to regenerate maize plants which were resistant to C. heterostrophus. The mitochondrial DNA isolated from resistant regenerates had altered restriction endonuclease patterns (Gengenbach et al. 1981; Kemble, Flavell and Brettel 1982). The mitochondrial synthesized polypeptides of these regenerates were also different relative to parental lines (Dixon et al. 1982).

Light and electron microscopy studies comparing pollen development in normal male-fertile and male-sterile cytoplasms have shown that in T and C cytoplasms the mitochondria of the tapetal cells degenerate early in pollen development (Lee, Gracen and Earle 1979; Lee and Warmke 1979; Warmke and Lee 1977; Warmke and Lee 1978). Male-sterile S cytoplasm had a normal pollen development until the later stages of development (Lee, Earle and Gracen 1980). This is with the gametophytic mode of restoration of S cytoplasm relative to the sporophytic restoration of C and T cytoplasms.

The cytoplasmic reversion of S cytoplasm to male fertility also implicates the mitochondrion in the cytoplasmic male sterility phenotype. The S1 and S2 (Pring et al. 1977) minilinear DNAs associated with S cytoplasm disappear in revertants to fertility and the principal genome undergoes rearrangement (Levings et al. 1980; Kemble and Mans 1983,. The evidence given above supports the conclusion that the mitochondrial DNA is responsible for several plant phenotypes.

Genome Structure

Mitochondria of all organisms have a similar function and perform the same fundamental set of reactions. The size of the mitochondrial genome can vary greatly. The vertebrate mitochondrial genome can range from 16.2 kilobase pairs (kb) to 17.5 kb, fungal mitochondrial genomes range from 17.3 kb to 100 kb, and higher plant mitochondrial genomes range from 135 kb to 2,400 kb (Sederoff 1984, Wallace 1982). The genome size anomaly among organisms and within higher plants is unexplained. The complete nucleotide sequence of human, bovine and mouse mitochondrial genomes have been determined (Anderson et al. 1981; Anderson et al. 1982; Bibb et al. 1981). The yeast mitochondrial genome is less conserved than that of the vertebrate mitochondria, and can vary in size and arrangement of genes (Clark-Walker and Sriprakash 1982). Filamentous fungi also show a range of sizes of mitochondrial genomes however, the genetic maps of Neurospora, Aspergillus and Podospora show some conservation when compared (Sederoff 1984).

Electron microscopy, restriction endonuclease analyses, and reassociation kinetics have been used to characterize the mitochondrial

genomes of plants. These studies led to the conclusion that the genome was circular, heterogeneous and much larger than that of animal and most fungal mitochondrial genomes (Quetier and Vedel 1977; Levings et al. 1979; Ward, Anderson and Bendich 1981). Restriction endonuclease profiles of normal cytoplasms and male-sterile cytoplasm of maize are distinct (Pring and Levings 1978), indicating that there is heterogeneity within maize.

Physical maps of the mitochondrial genomes for Brassica spp. and a normal fertile cytoplasm of Zea mays have been completed. The B. campestris mitochondrial genome consists of three circular chromosomes; the largest circle, termed the master chromosome, [218 kb] contains the complete mitochondrial genome and includes a 2-kb direct repeat (Palmer and Shields 1984). The two smaller circles [135 and 83 kb] each contain one copy of the 2-kb sequence and are postulated to be interconvertable with the master chromosome by intermolecular recombination within the 2-kb sequence. The physical map of B. oleracea is analogous to that of B. campestris. Both contain a master chromosome of 217 kb with a repeat within the genome which could result in the production of a 172 kb and a 45 kb circle through intramolecular recombination (Chetrit et al. 1984). However, another site of intramolecular recombination was found that resulted in the ribosomal RNA genes being separated by a variable distance.

The physical map of Z. mays is analogous to that of Brassica in that the genome consists of circles, but the map is more complex (Lonsdale, Hodge and Fauron 1984). The master chromosome is approximately 570 kb and includes six sequences which are repeated twice in the genome, five in a direct orientation and one in an inverted

orientation. Recombination between homologous repeats produces a series of sub-genomic circles which can undergo further recombination to produce a size range of related circles of 503 kb to 47 kb. The form inherited from one generation to the next is unknown. It may be a master chromosome which is converted to subgenomic circle by recombination during development or a set of preformed subgenomic circles. The function or advantage conferred by the repeats and a multipartite genome is also unknown.

An apparent exception to the circular form of the mitochondrial genome was observed in S cytoplasm of maize. The chromosome of the S cytoplasm exists in a flux between circular and linear forms (Schardl et al. 1984). The circular chromosome of the S cytoplasm contains sequences homologous to the terminal inverted repeated sequences of the episomal elements S1 and S2 (Pring et al. 1977). These sequences may undergo recombination with the autonomous S1 and S2 elements to produce a linear chromosome with S1 or S2 at their termini (Schardl et al. 1984). This type of linear chromosomal arrangement is not unique to the S cytoplasm of maize, in that some species of ciliated protozoans and yeast also have a linear mitochondrial genome arrangement (Sederoff 1984, Wallace 1982). However, no recombinational system has been described for producing these linear chromosomes.

Higher Plant Mitochondrial Minilinear and Minicircular DNAs

Vertebrate mitochondria have not been shown to contain minilinear or minicircular DNAs. However, many higher plants and fungi have been shown to contain a family of linear and circular double-stranded DNAs. These DNAs are distinguished from the principal genome by their smaller size and ability to replicate autonomously. The only exception may be Nicotiana and Oenothera where the mitochondrial genome may normally consist of circles in the range of approximately 10 to 30 kb .Dale 1981; Manna et al. 1985; Hiesel and Brennicke 1983). These small DNAs can be grouped on the basis of their homology to the principal genome and their linear or circular form.

The two best characterized minilinear DNAs are the episomal elements of S-male sterile cytoplasm S1 and S2 with molecular sizes 6,397 and 5,453 base pairs (bp), respectively. Both S1 (Paillard, Saderoff and Levings 1985) and S2 (Levings and Saderoff 1983) have been completely sequenced; both molecules have terminal inverted repeats of 208 bp which are identical on the two episomes. Proteins have been located covalently attached to the 5' ends of S1 and S2 (Kemble and Thompson 1982). These proteins are possibly involved with replication (Levings and Saderoff 1983]. S1 and S2 share 1462 bp of near perfect homology in addition to the identical 208 bp terminal repeats (Paillard, Saderoff and Levings 1985]. The sequence data have revealed two open reading frames in S2 designated URF-1 [3313 bp] and URF-2 [1017 bp]. The URF-2 is present in S1 which also has URF-3 [2787 bp] and URF-4 [768 bp]. These unidentified open reading frames may play a role in replication and maintenance of the S1 and S2 molecules

Although S1 and S2 are found as autonomous DNAs in the S-sterile cytoplasm, they have extensive homology to the principal genomes of the N (Lonsdale, Thompson and Hodge 1984) and S (Schardl et al. 1984) cytoplasms. There is slight homology to S1 and S2 in the T and C cytoplasms (Thompson, Kemble and Flavell 1980; Spruill, Levings and Sederoff 1980, Koncz et al. 1984). The cytoplasmic reversion of an S cytoplasm to male fertility has been associated with the loss of S1 and S2 and rearrangement of the principal genome in regions sharing homology with S1 and S2 (Levings et al. 1980). Molecular studies on the S-cytoplasm revertants have shown a recircularization of the principal genome and the deletion of the integrated terminal-inverted repeat of S2 [Schardl, Pring and Lonsdale 1985 in press]. The loss of the integrated inverted repeat was correlated with the loss of URF-1 from the S2 sequences in the principal genome. These changes may be involved with the reversion of the cytoplasm to male fertility.

The S1 and S2 episomes are similar to two pairs of linear DNAs designated R1 [7.4 kb] and R2 [5.4 kb], found in RL cytoplasms of Latin American races of maize and D1 [7.5 kb] and D2 [5.5 kb] found in Zea diploperennis (Weissinger et al. 1982; Weissinger et al. 1983, Timothy et al. 1983). S2, R2 and D2 are apparently identical by heteroduplex analysis. A recombination event between R1 and R2 could produce a molecule similar to S1 (Timothy et al. 1983). Kemble and Bedbrook (1980) described two smaller linear DNAs of 2.3 and 2.1 kb found in N, S, C cytoplasms, and T cytoplasm, respectively. The 2.3 and 2.1 kb DNAs are homologous to each other (Kemble and Thompson 1982).

Minilinear DNAs have also been found in other genera. Two minilinear DNAs N1 [5.7 kb] and N2 [5.3 kb] are present in the

male-sterile cytoplasm IS1112C of Sorghum bicolor (Pring et al. 1982, Dixon et al. 1982; Leaver et al. 1982). These DNAs were thought to be analogous to the S1 and S2 molecules of maize. However, recent studies have shown that N1 and N2 do not hybridize to the sorghum principal mitochondrial genomes tested, S1, or S2 (Chase and Pring 1985a). Palmer et al. [1983] described a linear DNA 11.3 kb in size from B. campestris and B. napus which is associated with cytoplasmic male sterility. This minilinear DNA can vary in copy number, has no internal repeats and is not homologous to the principal genome of Brassica mitochondria or the S1 and S2 DNAs.

The mitochondria of maize contain minilinear molecules which are single or double stranded RNA. Sisco et al. (1984) described two double-stranded RNAs associated specifically with the cytoplasmic male sterile maize W182BN[LBN]. These RNAs were designated LBN-1 and LBN-2 and are 2.8 kb and 0.76 kb respectively. The RNAs are maternally inherited, are not associated with virus-like particles, do not show virus-like transmission and cannot be translated in vitro. The Rf genes have no effect on their presence, but the nuclear background affects the copy number of the RNAs. These double-stranded RNAs are related to two single stranded RNAs found in the Ru, S, Vg and a fertile cytoplasmic revertant of Vg [L369) cytoplasms (Schuster, Sisco, and Levings 1983). The single-stranded RNAs are designated S/Ru-RNA-a and S/Ru-RNA-b and are 2.9 kb and 0.75 kb respectively. The S/Ru-RNA-b hybridizes to the LBN-1 and LBN-2 RNAs, but does not hybridize to any mitochondrial DNA.

The minilinear DNAs have the common features of being consistently found associated with the mitochondria of higher plants, capable of autonomous replication, maternally inherited, and lacking a definite

phenotype. Although free S1, S2, and the Brassica minilinear DNA are associated with male-sterile cytoplasms, there is no defined gene or gene product of any of the minilinear DNAs which has a phenotype.

Many minicircular DNAs have been described as being part of the mitochondrial genome of higher plants. The descriptions vary from electron microscopy estimates of size and frequency to molecular characterization. In pea 1% of the genome is in the form of circular molecules which ranged from 3 to 5 micrometers (Kolodner and Tewari 1972 . The mitochondrial genome of Glycine max was grouped into seven size classes ranging from 5.9 micrometers to 29.9 micrometers, however circles less than 3 micrometers were seen and were classified as minicircles (Synenki, Levings and Shah 1978). Levings et al. (1979) described the distribution of circular DNA molecules in the maize cytoplasms N, T and S. The most frequent classes were 21 micrometers (N, at 46%, 17 micrometers (S) at 40%, and 25 micrometers (T at 47%. However, smaller circles were noticed but frequencies were not calculated. A circle of 1.3 megadaltons [approximately 1.95 kb] was found in N, T, and S cytoplasms and a circle of 0.6 megadaltons [approximately 0 9 kb] was found only in the S cytoplasm. These families of circular and linear molecules were later used to distinguish the various cytoplasms of maize [Kemble and Bedbrook 1980). In their study agarose gel electrophoresis showed that all maize mitochondria contained a high molecular weight DNA corresponding to the principal genome and a 1.94 kb DNA. The cytoplasms could be distinguished by a 2.3 kb DNA in N, C and S cytoplasms, a 2.1 kb DNA in T cytoplasms, a 1.57 kb and 1.42 kb DNA only in C cytoplasms and S1 and S2 in only S cytoplasms.

In a cell suspension of Black Mexican maize (cms N cytoplasm), two supercoiled minicircular DNAs were identified of 1.5 and 1.8 kb (Dale 1981). When purified by agarose gel electrophoresis these DNAs hybridized to each other, to a multimeric set of molecules, and to two RNA transcripts (Dale 1981). Dale, Dussing and Keene (1981) examined the mitochondrial DNAs from cell cultures of Nicotiana tabacum Wisconsin 38, N. rustica, Datura stramonium, var. white inermis, Solanum dulcimara, and Phaseolus vulgaris. All the cultures contained circular DNAs which were heterogeneous in size. The P. vulgaris culture contained a group of supercoiled minicircles that were shown to be multimers of a predominant 1.9 kb circle (Dale 1981). The two cultures of N. tabacum, differing only in the time they had been in culture, had distinct frequencies and size classes of circular DNAs. The restriction endonuclease profiles were changed in one or two sites. The mitochondrial genome had presumably undergone rearrangements or the explant sources of the two cultures were different (Dale, Wu and Kiernan 1983).

The supercoiled DNA fraction from mitochondria purified from cell cultures of Oenothera berteriana had a large number of minicircular DNAs which could be separated by agarose gel electrophoresis (Brennicke and Blanz 1982). The size classes were 6.3, 7.0, 8.2, 9.9, and 13.5 kb, with larger DNAs also being present. As determined by restriction endonuclease assay they were homogeneous within the classes, but generated independent patterns among the classes, indicating that the circles were not related. However, when the gene for cytochrome oxidase subunit two was cloned from O. villaricae and used as a probe, it hybridized to circles from five size

classes (Hiesel and Brennicke 1983). This may indicate that there is homology among some of the larger minicircles and a structural gene of the principal genome of O. villaricae.

In a survey of 13 cytoplasmic-sterile and five fertile varieties of Beta vulgaris a series of minicircular DNAs were designated "a" [1.5 kb], "b" [1.45 kb], "c" [1.4 kb], and "d" [1.3 kb] (Powling 1981). All of the sterile varieties contained only the 1.5 kb minicircular DNA with the exception of line 01 I13M4, which also contained a 7.3 kb supercoiled DNA. The fertile varieties contained minicircular DNAs of 1.4 kb, 1.3 kb, and either 1.5 kb or 1.45 kb. The normal and cytoplasmic male sterile types could also be distinguished by restriction endonuclease profiles (Powling 1982). Molecular cloning and cross hybridization of the minicircular DNAs showed that none of the DNAs had homology to any other mitochondrial DNA, including the high molecular weight DNA, except that the 1.5 and 1.4 kb DNAs, cross-hybridized (Powling and Ellis 1983). A 1,440 bp minicircular DNA was isolated from normal sugar beet, the sequence was determined and three RNA transcripts were characterized (Hansen and Marcker 1984). The major transcript was 850 bases, the two minor transcripts were 1100 and 950 bases. All of the reading frames of the minicircle contained numerous stop codons.

Two minicircular DNAs were detected in mitochondria of the male-sterile IS112C cytoplasm of Sorghum bicolor (Chase and Pring 1985b). The two minicircular DNAs did not hybridize with each other, to chloroplast DNA, or to nuclear DNA. The 2.3 kb minicircle did hybridize to the 1.9 kb maize minicircle. Homologies to both molecules were

detected in mitochondrial RNA. The distribution of these minicircles was variable with no apparent correlation to male sterility.

Similar studies of minicircular DNAs in Vicia faba have shown the presence of three covalently closed circular DNAs. Two distinct minicircles of 850,000 Daltons and a minor minicircle of 750,000 Daltons (Negruk, et al. 1982). Boutry and Briquet (1982) separated five discrete bands in agarose gels of V. faba mitochondrial DNA which could be used to distinguish the male-sterile cytoplasms. In a survey of species related to V. faba (Nikiforova and Negruk 1983), minicircular DNAs were found in V. villosa and Medicago sativa. However, V. sativa had no minicircular DNA less than 8 to 10 kb. Also surveyed were root, leaf, stem and 12 year old cell culture tissues of V. faba; these tissues had no noticeable differences in the pattern of small mitochondrial DNAs, which indicates an inherent stability of these minicircular DNAs. Three minicircular DNAs were cloned from V. faba var. Russian Blacks designated ccc1A, ccc1B and ccc2 (Negruk et al. 1985). Hybridization studies using the cloned DNAs showed that ccc1A was homologous to both ccc1B and ccc2, however ccc1B was not homologous to ccc2. The minicircular DNAs were present in high molecular weight DNA as both multimers and integrated forms.

In separate experiments four minicircular DNAs were cloned from V. faba normal and cytoplasmic sterile cytoplasms and homologies were determined (Goblet, Flamand and Briquet 1985). The two largest minicircles isolated from a sterile cytoplasm (1.7 S) and a fertile cytoplasm (1.7 F) were the same size (1.7 kb) and hybridized to each other, but varied in their restriction endonuclease maps. The 1.7 F minicircle was in all cytoplasms tested, whereas the 1.7 S minicircle

was only associated with cytoplasmic male sterile lines. A 1.54 kb minicircle which hybridized to the 1.7 S minicircle had the same arrangement of restriction endonuclease sites and was found in only the 350 type cytoplasmic male sterile line. This minicircle is thought to have been derived from the larger sterile specific minicircle which is also present in cytoplasm 350. The 1.42 kb minicircle which was found in all cytoplasms tested had a unique restriction endonuclease profile, weak hybridization to the sterile specific minicircles and stronger hybridization to the 1.7 F minicircle.

In an electron-microscopy study of Citrus reticulata, var. Clementino, C. limonum, var. Verna and three varieties from C. sinensis, the bulk of the circular mitochondrial DNA was in the size range of 0.3 micrometers to 20 micrometers with the most frequent class being less than 10 micrometers in size. The histograms of linear molecules from the Navelino and Verna varieties had peaks at 0.45 micrometers and 1.55 micrometers respectively (Fontarnau and Hernandez-Yago 1982). Theoretically, the histograms of mitochondrial DNA could be used to distinguish or group citrus cytoplasms. Minicircular DNAs were observed in mitochondria from eight Triticum and Aegilops species, the sizes for the small circles ranged from 0.1 to 2.0 micrometers with a model length of 0.2 to 0.4 micrometers (Handa et al. 1984). These lengths were similar for all wheat cytoplasms examined. A 1.45 kb minicircular DNA was found in mitochondria isolated from Fertile Helianthus annuus, but not the cytoplasmic sterile H. annuus (Leroy et al. 1985).

As with the minilinear molecules found within the mitochondria, the minicircular molecules have not been definitively associated with any phenotype. Their distribution seems random among cytoplasms and their

existence is usually thought to be dispensable. Their ability to autonomously replicate ensures their segregation and retention during cell division. Those minicircular DNAs having homology to the principal genome may be derived from the principal genome by some type recombination, or excision event. The same may be true for those lacking homology to any other mitochondrial DNA, but their sequences diverged so they no longer have common sequences or they may have an origin which is not mitochondrial.

Minicircular DNAs are found frequently in fungal mitochondria, many are distinguished from the plant minicircles in that they are associated with a defective phenotype. Examples are the minicircular-mitochondrial DNAs of the Saccharomyces petite mutants, Aspergillus ragged mutants, Podospora senescence, and Neurospora stopper mutants (Sederoff 1984; Esser et al. 1983). The petite S. cerevisiae are characterized as being cytoplasmically-inherited respiratory deficient mutants, with large deletions in their mitochondrial genomes. The mitochondrial genome may be completely deleted or part may remain as an amplified series of repetitive sequences (Locker, Lewin and Rabinowitz 1979; Bernardi et al. 1980). The minicircular DNAs of the petite mutants are the excised portion of the mitochondrial genome and they are homologous to the wild type mitochondrial genome. Since members of the genus Saccharomyces are facultative anaerobes, they survive without functioning mitochondria but form a smaller colony on solid media relative to the grande (wildtype mitochondrial genome) Saccharomyces. The petites are classified on the basis of transmission when crossed with grande Saccharomyces. Neutral petites do not transmit the petite phenotype, while suppressive petites do.

The ragged, senescence and stopper phenotypes formed in Aspergillus, Podospora anserina and Neurospora crassa, respectively, are analogous to the petite mutants except that these genera are obligate aerobes so the loss of mitochondrial function results in irregular growth phenomena. The loss of mitochondrial function is due to the excision and amplification of a sequence of mitochondrial DNA which suppresses the normal mitochondrial genome. The regions of the wild-type genome which are excised have been mapped to several regions for some of these mutants. There are regions which are excised and amplified more frequently. The area from which the amplified DNA is excised and the nuclear genotype of the mutant will determine the petites' individual characteristics (Lazarus and Kuntzel 1981, DeZamaroczy et al. 1981; Belcour et al. 1981; Kuck, Stahl and Esser 1981; Wright, Morrum and Cummings 1982; Bertrand et al. 1980, de Vries et al. 1981).

As a consequence of senescence the nuclear genome of P. anserina gains multiple or single copies of an amplified senescent DNA designated senDNA. The nuclear genome of the young nonsenescent mycelium does not contain any copies of this sequence. It is believed that senDNA somehow moves to and integrates within the nuclear genome during the excision and amplification of this mitochondrial sequence. A mutant max^{1} (Vierny et al. 1982) which escapes senescence, is characterized by a deletion of the senDNA from the mitochondrial genome; this mutant was shown to contain a single copy of senDNA in the nuclear genome of young mycelium [Wright and Cummings 1983a]. The senDNA is homologous to an intron of the cytochrome c oxidase subunit two gene, this DNA is transcribed into two RNAs [2.5 and 2.4 kb]. These RNAs are hypothesized to be from the

integrated and autonomous forms, respectively, of senDNA, and they may play a role in senescence of Podospora or excision of the senDNA from the principal genome Wright and Cummings 1983b; Osiewacz and Esser 1984; Kuck et al. 1985).

Four minicircular DNAs have been found in Neurospora which are not associated with any phenotype and have characteristics distinct from the minicircles described above. They are maternally inherited, located within mitochondria and have little or no homology to and are not suppressive of the principal mitochondrial genome. The minicircle designated Mauriceville was isolated from N. crassa strain Mauriceville-1C [FGSC#2225) and has a monomer size of 3,581 bp. Like the fungal minicircles described earlier it is amplified as multimers but does not appear to affect the growth rate of the N. crassa in which it is found. The transcript from the minicircle is 3.3 to 3.4 kb, and a mitochondrial gene product of molecular weight 41,000 to 46,000 has been associated with mitochondria containing this DNA [Collins et al. 1981,. A group of Pst I sites were located on the minicircular molecule which resemble the GC-rich palindromic sequences which flank many genetic elements in Neurospora (Nargang et al. 1983). The open reading frame detected on the Mauriceville minicircle has a codon usage and the conserved sequence elements of a group I mitochondrial intron (Michel, Jacquier and Bernard 1982) which suggests that the minicircular DNA may be an escaped intron [Nargang et al. 1984,. Another minicircular DNA of Neurospora was isolated from N. intermedia strain Varkud-1C. It was homologous to Mauriceville (Nargang et al. 1984). Neurospora crassa strain (Fi,1 N6-5) and N. intermedia strain (P405 Labelle) contained minicircles which only weakly hybridize to Mauriceville or any

mitochondrial RNAs (Stohl et al. 1982). These autonomously replicating DNAs of Neurospora may represent a unique family of mitochondrial genetic elements which have characteristics that are very similar to some of the higher plant mitochondrial minicircles.

Two minilinear DNAs have been isolated from Clavicaps purpurea. These DNAs were free in the mitochondria and were also integrated into the principal genome (Tudzynski, Duvell and Esser 1983). A minicircular DNA was isolated from the plant pathogen Cochliobolus heterostrophus, this DNA was found autonomously as monomers and head-to-tail multimers and was integrated into the principal genome of the mitochondria which either did or did not carry the autonomous DNA (Gerber, Turgeon and Yoder 1984).

Mitochondrial DNA Replication

In order for the mitochondrial DNA to be inherited it must be replicated so the genome is not diluted as the organelle divides. The study of organelle heredity has led to the discovery that in most cases the inheritance of the mitochondrial genome is uniparental and vegetatively segregated (Birky 1983). The study of mitochondrial replication has been most complete in Saccharomyces and vertebrate cell culture. The lack of information on plant mitochondrial DNA replication is a reflection of the large size and complex heterogeneity of the genome.

It has been shown that the mitochondrial DNA replication of Saccharomyces is independent of nuclear DNA replication and occurs throughout the cell cycle (Blanc and Dujon 1982). Analysis of the independent petite mutants has identified areas of the wild type genome

which have been associated with the ability to initiate replication. These areas have the ability to maintain or amplify the excised portion of DNA of the petite mutation. The location, orientation and primary structure of these mitochondrial sequences have been determined. They are characterized by their expected presence in the majority of repeating units of the petite mutants, the correlation of a particular sequence or change in that sequence with the behavior of a petite mutation, and the *in vivo* and or *in vitro* identification of origins of DNA replication.

Baldacci, Cherif-Zahar and Bernardi (1984, have directly demonstrated two origins of replications (ori) on the genome of *S. cerevisiae*. They identified the location of the RNA primer which initiates bidirectional DNA synthesis on both strands of the ori. By comparing many petite mutants they have constructed a conserved sequence for seven identified oris. From 5' to 3' the conserved ori sequence consists of three GC clusters alternating with long AT stretches; the terminal AT cluster is interrupted by another GC cluster in ori 4, 5, and 7.

Hypersuppressive petite mutants are defined as transmitting the petite phenotype to 95% of the progeny when crossed to a yeast with a wild type mitochondrial genome. The hypersuppressive mutants contain amplified tandem repeats of approximately 1% of the wildtype mitochondrial genome. The amplified sequence is able to replicate in preference to the wildtype genome (Blanc and Dujon 1980). When the repeating units of many hypersuppressive mutants were compared, a 300 bp conserved sequence was identified in all the mutants (Dujon and Blanc 1980). The conserved sequences were designated rep sequences. Each rep

sequence cross hybridized to all the other rep sequences isolated on independent hypersuppressive mutants but were classified into three groups based on the principal genome sequences which flanked the rep sequence (Blanc and Dujon 1982). Having the rep sequence amplified by tandem repeats on the single petite genome gives it a selective advantage relative to the wildtype genome which has three rep sequences.

When less suppressive mutants were isolated from hypersuppressive mutants the sequences which where retained by the petite mutants were changed. Two mutants were characterized which retained mitochondrial genomes consisting of only adenine and thymine base pairs. Although suppression was reduced these genomes were able to replicate, indicating that an ori or rep sequence is not an obligate component of a yeast mitochondrial replicon (Fangman and Dujon 1984).

As in yeast, the replication of the vertebrate mitochondrial DNA is not linked to the replication of nuclear DNA. Throughout the cell cycle molecules are selected at random from the population for replication (Bogenhagen and Clayton 1977). There are two origins of replication for the unidirectional synthesis of mammalian mitochondrial DNA. One origin located in the displacement loop for heavy strand synthesis and another origin located two-thirds around the genome where light strand synthesis is initiated (Clayton 1982). Synthesis is initiated by an RNA primer whose promoter (LSP) is the same as that used to generate the polycistronic messenger RNA (Chang and Clayton 1985). Specific processing generates the primer for initiation of mitochondrial DNA synthesis rather than production of mitochondrial transcripts. Light strand synthesis does not occur until the heavy strand nascent DNA

reaches the second origin of replication where the unidirectional synthesis of the light strand begins.

MATERIALS AND METHODS

Maize Cell Suspension Culture Growth and Nucleic Acid Labelling

The cell suspension culture of Black Mexican maize was maintained as described by Chourey and Zurawski (1981). The growth curve of the cell suspension was determined over a 15 day period after subculturing by measuring the increase in dry weight of the cell culture. Normally the suspension was subcultured every seven days. Stationary and logarithmic phase cultures were defined as those cultures which were 14 and 7 days post subculturing, respectively. Lag phase cultures were produced by removing cells from stationary phase cultures, washing once with new medium by centrifugation at 100 g for 5 min, and resuspending the cells in four times the original volume with new medium. Cell viability was determined by staining with fluorescien diacetate and viewing with ultraviolet illumination.

Nucleic acids of the cell suspension were labelled by the addition of 15 microcuries per milliliter of phosphorus 32 (as orthophosphate in aqueous solution from Amersham) into the culture and allowing the culture to grow 24 hours prior to harvesting the DNA. Nucleic acid was labelled at various points during the growth phases of the cell suspension. The cells were harvested and the DNA extracted as described below.

Isolation of Maize DNA and RNA

Maize mitochondrial DNA was isolated by a modification of the method described by McNay, Chourey and Pring [1984]. The homogenization buffer consisted of 0.3 M mannitol, 3 mM disodium-EDTA, 5 mM diethyldithiocarbamate, 5 mM mercapto-ethanol, 0.1% w/v bovine serum albumin and 0.05 M Tris-HCl, pH 8.0. Green tissue was homogenized with three 5 sec bursts in a Waring blender. The homogenate was filtered through miracloth and centrifuged at 100 g for 10 min; this pellet consisted of nuclei and cell debris. The supernatant was centrifuged at 1,000 g for 10 min, the 1000 g pellet was resuspended in homogenization buffer and centrifuged at 1,000 g for 10 min. The 1000 g pellet consisted primarily of chloroplasts when green tissue was used. The first supernatant from the 1,000 g pellet was centrifuged at 16,000 g for 10 min to pellet mitochondria. The 1000 g and 16,000 g pellets were resuspended in a solution of 0.3 M sucrose, 10 mM magnesium chloride, 50 mM Tris-HCl, pH 7.5, and 0.02 mg/ml w/v deoxyribonuclease I and incubated for 60 min at room temperature and 4 C, respectively. The 100 g pellet was resuspended directly in lysis buffer and was not digested with deoxyribonuclease. After digestion, the chloroplast and mitochondrial fractions were layered over a solution of 0.6 M sucrose, 20 mM disodium-EDTA, 0.01 M Tris-HCl, pH 7.2 and centrifuged at 1,000 g and 12,000 g, respectively, for 20 min. The pellets were resuspended in the underlayer solution and centrifuged for 10 min at 1,000 g and 16,000 g for chloroplasts and mitochondria, respectively. These pellets and the 100 g pellet were lysed in a solution of 50 mM Tris-HCl, pH 7.5, 20 mM disodium-EDTA [NN] plus 0.1 mg/ml proteinase K and 0.5% w/v sodium dodecylsulfate at 37 C for 60 min. The lysates were phenol extracted

twice and ethanol precipitated with 0.1 volume of 8 M ammonium acetate and 2 volumes of absolute ethanol at -80 C for 60 min. The precipitates were collected by centrifugation at 16,000 g for 15 min, washed in 70% ethanol and vacuum dried. DNAs were resuspended in 0.1X NTE (1 mM NaCl, 0.1 mM disodium-EDTA and 1 mM Tris-HCl, pH 8.0).

Mitochondrial RNA was purified from mitochondria isolated as described above. All buffers contained 1 mM aurintricarboxylic acid. No deoxyribonuclease digestion was performed and lysis buffer did not contain proteinase K. Immediately after lysis four phenol extractions were performed, followed by an extraction with 24:1 chloroform:isoamyl alcohol. One gram of CsCl was added to the aqueous phase and the solution was brought to 2.5 ml with NN. This solution was layered over 1.5 ml of 7.5 M CsCl made in NN in a Beckman SW 50.1 tube; tubes were completely filled with sterile mineral oil and centrifuged 12 hr at 95,000 g. The RNA pellet was rehydrated in NN and stored as an ethanol precipitate at -80 C.

Nuclear DNA for hybridization with cloned mitochondrial probes was prepared by a modified procedure of Kislev and Rubenstein (1980). Green-house grown tissue 3-5 weeks old was homogenized for 30-90 sec in a Waring blender with liquid nitrogen. All further manipulations were performed at 4 C. The powdered tissue was added slowly to 200-300 ml of MNIB [0.4 M sucrose, 2 mM calcium-chloride, 2% w/v gum arabic, 4 mM n-octylalcohol, 2 mg/ml polyvinlypyrrolidone, 0.4 mg/ml Ethidium bromide and 20 mM Tris-HCl, pH 7.6) with constant stirring. The tissue was infiltrated by applying a vacuum of 15 in Hg for 15 min, extracted in MNIB with a Polytron homogenizer at maximum speed for 30 sec, and filtered through a 74 micrometer nylon mesh. The residue was

re-extracted, filtered, and the filtrates were combined and filtered through a 44 micrometer nylon mesh and centrifuged at 1,000 g for 10 min. The pellets were resuspended in NSB (0.2 M Sucrose, 2 mM calcium chloride, 10 mM Tris-HCl, pH 7.4 and 0.15% v/v Triton X-100) and centrifuged 10 min at 1,000 g. This step was repeated until the pellets had lost their green color, or a maximum of four times. The last pellet was resuspended in 3 ml of PDM (10 mM NaCl, 10 mM disodium-EDTA, 0.5% sodium dodecylsulfate, 0.01 M Tris-HCl, pH 8.6 and 0.2 mg/ml proteinase K). The solution was incubated for 18 hr at 37 C and then centrifuged at 12,000 g for 20 min. The supernatant was phenol extracted three times and dialyzed 48 hr against several changes of 0.1X NTE.

Molecular Cloning of Mitochondrial Minicircular DNAs

Minicircular DNAs were purified from other mitochondrial DNAs by preparatory agarose gel electrophoresis. The DNAs were extracted from the gel with NA-45 DEAE cellulose membrane (Schleicher & Schuell, Inc.) as per manufacturer's instructions. Restriction endonucleases and T4 DNA ligase were purchased from BRL laboratories and used as the manufacturer instructed. The 1.9 kb DNA was restricted with Bam HI and ligated with Bam HI restricted pBR322 or pUC8 DNA. The 1.4 kb DNA was restricted with Eco RI and ligated with similarly cut pUC8 DNA. The ligated DNAs were transformed into Escherichia coli strain HB101 (for pBR322) or strain JM83 T8I (for pUC8, obtained from T. D. Baldwin). Transformation mixtures of pBR322 were plated on LB-agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl, 10 g agar/L) with 100 mg ampicillin/L. Colonies were counter-selected on LB-agar plates with 20 mg tetracycline/l. Those colonies which grew on ampicilin but not

tetracycline were selected as recombinants. Transformation mixtures of pUC8 were plated on LB-agar plates with 100 mg ampicillin and 40 mg Xgal/l. The white colonies were selected as recombinants. After selection, all recombinants were verified by hybridization to total mitochondrial DNA.

Subclones for sequencing the 1.4 kb minicircular DNA were made by purifying and extracting the 1.4 kb Eco RI restriction fragment from the recombinant clone as described above. Both orientations of the full length 1.4 kb mitochondrial DNA in M13mp8 was used to produce truncated clones by exonuclease III and exonuclease VII treatment as described by (Yanish-Perron, Vieira and Messing 1985).

Electrophoresis and Hybridizations

Mitochondrial DNAs were electrophoresed in 0.7% w/v agarose gels at 1.6 V/cm using TPE buffer (1 mM disodium-EDTA, 36 mM disodium-phosphate and 30 mM Tris-HCl, pH 7.8). DNA was immobilized on nitrocellulose filters by the method of Southern (1975). Prehybridization was at 66 C for 1 hr in 10X Denhardt's solution (0.1% sodium dodecylsulfate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.2% bovine serum albumin, 0.1 mg/ml sheared-heat-denatured salmon sperm DNA) in 3X SSC (0.45 M NaCl, 45 mM Na-citrate). The prehybridization solution was discarded and hybridization solution having the same constituents plus radioactive labeled probe was added and incubated 16 hr at 66 C. The radioactive probes were made by nick translation (Rigby et al. 1977) and unincorporated label was removed from DNA by chromatography through Sephadex G-50. Filters were twice washed 15 min each with 65 C 3X SSC and once in 65 C 0.3X SSC, vacuum dried and autoradiography performed.

Mitochondrial RNA from the ethanol precipitation was collected by centrifugation in a microfuge. The pellet was washed once with 70% ethanol and vacuum dried. The pellet was rehydrated on ice in 10 mM disodium-phosphate, pH 7.0. This RNA was glyoxylated, electrophoresed in 1.6% w/v agarose gels (0.01 mg RNA/lane), and transferred to nitrocellulose filters by the methods of Thomas (1983). Filters were hybridized with M13mp8 strand specific probes produced by the method of Hu and Messing (1982), or the nick translated rDNA probes obtained from D. M. Lonsdale (Stern, Dyer and Lonsdale 1982). The same prehybridization and hybridization conditions were used as the DNA filters. Filters were washed with four changes of 2X SSC plus 0.1% w/v sodium dodecylsulfate for 5 min each at room temperature and twice with 0.1X SSC plus 0.1% w/v sodium dodecylsulfate for 15 min each at 50 C.

Sequencing of the 1,445 bp Minicircular DNA

The M13mp8 subclones which represented the entire length and both strands of the 1,445 bp DNA were sequenced by the chain termination method of Sanger, Nicklen and Coulson (1977) as modified by Yanish-Perron, Viera and Messing (1985). Sequence reactions were electrophoresed on 6% to 8% acrylamide gels which were 0.2 mm to 0.4-0.8 mm thick from top to bottom according to the methods of Olsson et al. (1984). The sequence data were analyzed using the DNA and protein sequence analysis program for the Apple II computer written by Larson and Messing (1983).

RESULTS

In Vivo Labelling of Mitochondrial DNA

A typical growth curve for the Black Mexican cell suspension used in this study (Fig. 1) shows that early stationary phase was reached approximately 12 days after subculturing. A similar growth curve for this cell line has been described previously (Chourey and Zurawski 1981). The cells from the stationary phase were viable as determined by staining with fluorescien diacetate. Fluorescence of these cells was decreased when compared to that of cells from logarithmic phase cultures (Fig. 2, plates A and B), but the fluorescence characteristic of cells in logarithmic phase was regained when stationary phase cultures were subcultured into fresh medium and allowed to grow for several days.

The mitochondrial genome of Black Mexican maize consisted of a large molecular weight principal genome and a group of small DNAs with a much higher electrophoretic mobility (Fig. 3, lane 2). Exonuclease III treatment digested the three upper bands of the small DNAs, but not the the lowest two bands (Fig. 3, lane 1). Therefore the upper bands were open-circle or linear and the lower bands were covalently closed circular (ccc) DNAs (Fig. 3, lane). The uppermost band of the fast migrating group consisted of the single form of the 2.3 kb mini-linear DNA. The other bands were the three forms of the 1.9 and 1.4 kb minicircular DNAs. The three forms of each minicircular DNA were, form I (ccc, with the fastest mobility, form II (open circle), and form III

(linear had slower mobilities than the Form I DNA. The Form II DNA had a slightly slower mobility than Form III DNA.

Logarithmic and stationary phase cultures (6 and 14 days post subculturing respectively) as well as lag cultures which had been in new medium for 0, 1, 2, 4, and 6 days were incubated with ^{32}P for 24 hours. The mitochondrial DNA from all growth phases of the cell suspension, and mitochondriel DNA isolated from Black Mexican Sweet whole plants had the same components by ethidium bromide staining (Fig. 4). There were some differences among preparations in the amount of degradation which occurred during the extraction. Mitochondrial DNA extracted from stationary phase cultures had the most degradation.

The relative copy number of the 1.9, 1.4 and 2.3 kb DNAs of green plants and cell suspension was determined during various growth phases from laser densitometer scans of ethidium bromide stained agarose gels of mitochondrial DNA. The area under the 2.3 kb DNA peak was assigned a copy number of one. The area of all the small DNA peaks were used to calculate the copy number of the 1.4 and 1.9 kb DNAs relative to the 2.3 kb DNA peak after correction for kb differences.

The calculated copy numbers for the small DNAs isolated from green plants were 1.03, 0.80 and 1 for the 1.9, 1.4 and 2.3 kb DNAs, respectively (Fig. 4, lane 1). The 1.9 and 1.4 kb minicircular DNAs were in higher copy number in mitochondrial DNA isolated from cell suspensions in logarithmic phase. The relative copy numbers of the 1.9, 1.4 and 2.3 kb DNAs were 1.49, 1.42, and 1, respectively (Fig. 4, lane 2). This represents a 140% increase in the 1.9 kb minicircle and a 78% increase in the 1.4 kb minicircle in the cell suspension. The 1.9 kb minicircle greatly increased in copy number relative to the other small

DNAs in the first few days of lag phase. The 1.9 kb minicircle increased 920%, to a copy number of 7.80 relative to the 2.3 kb DNA [Fig. 4, lane 4] during this period.

The 1.4 kb minicircular DNA also increased 140% to a relative copy number of 2.07 in day 1 lag cells. The initial amplification of the minicircular DNAs of early lag phase did not continue through lag phase As cells progressed into lag phase the amplified copy number of the 1.9 and 1.4 kb minicircles was not sustained (Fig. 4, lanes 4-8). When the lag phase cultures had been in new medium for 7 days the stoichiometries of the small DNAs approached that of logarithmic cultures Fig. 4, lane 8). The relative copy numbers of the 1.9, 1.4 and 2.3 kb DNAs were 1.65, .61 and 1, respectively. The same group of minicircular and minilinear DNAs were detected in whole plants, stationary phase, lag phase and logarithmic phase cultures. No other small DNAs were detected in any of the mitochondrial DNA preparations.

Synthesis of DNA during the various culture phases was followed by ^{32}P incorporation into mitochondrial DNA. All mitochondrial DNAs incorporated large amounts of ^{32}P during logarithmic phase, whereas there was no detectable incorporation into mitochondrial DNA from stationary phase cultures over a 24 hr labelling period (Fig. 5, lanes 1 and 2). The 1.9 kb minicircular DNA which was present in greater amounts in the early lag phase cultures, was also more intense in the corresponding lanes of the autoradiogram (Fig. 5, lane 3 . The enhanced incorporation of isotope into the 1.9 kb minicircle DNA during the first 24 hrs in new medium indicates the increased copy number of the 1.9 kb minicircle, was due to synthesis of 1.9 kb DNA and not degradation of the other two DNAs. Enhanced incorporation of isotope into the 1.9 kb

minicircle no longer occurred in late lag phase cells (Fig. 5, lanes 5-7).

The 1.4 and 2.3 kb DNAs also incorporated isotope during the first 24 hrs of lag phase (Fig. 5, lane 3), but much less than the incorporation into the 1.9 kb minicircle. The 1.4 and 2.3 kb DNAs did incorporate isotope during the later 24 hr periods of lag phase (Fig. 5, lanes 4-7). This reflects the shift in copy number of the small DNAs during the last days of lag phase becoming more like logarithmic phase cells.

The principal genome of mitochondria was examined by digesting the mitochondrial DNA with Bam HI. Mitochondrial DNA isolated from cells in all of the growth phases was composed of the same set of restriction endonuclease fragments as seen by the ethidium bromide stained lanes (Fig. 6, lane 2, 4, 6, 8, 10, and 12). The ethidium bromide stained, and corresponding autoradiogram of mitochondrial DNA isolated from stationary phase cells showed that DNA from the principal genome was present in stationary phase cells, but none of the restriction fragments incorporated isotope over the 24 hr labelling period (Fig. 6, lanes 2 and 3). The ethidium bromide stained lane and corresponding autoradiogram of restricted mitochondrial DNA from logarithmic cells indicated that stationary phase and logarithmic cells had the same mitochondrial DNA restriction patterns. However, there was a large amount of incorporation into the principal genome of logarithmic cells relative to no incorporation in stationary phase cultures (Fig. 6, lanes 3 and 5).

The first 24 h in lag phase showed enhanced incorporation into the 1.9 kb minicircular DNA and a subset of restriction fragments (Fig 6

lanes 4 and 5). This banding pattern was identical to the restriction profile of the chloroplast genome (Fig. 6, lane 1,. The first DNAs to show incorporation as synthesis was renewed were the small mitochondrial DNAs and the genome of the proplastids. Apparently the stationary phase cultures renew DNA synthesis first in the proplastid and the 1.9 kb minicircle DNA. As lag phase progressed the restriction fragments of the principal mitochondrial genome began to incorporate 32P (Fig. 6, lanes 6-13,. Synthesis of the principal genome begins after a significant amount of synthesis has occurred in the 1.9 kb minicircle and in the proplastid DNA.

Incorporation of 32P into nuclear DNA was also examined over this time period. The nuclear DNA from stationary phase or one day into lag phase did not incorporate 32P Fig. 7, lane 1-4 . The nuclear DNA extracted from two days into lag phase had a large amount of 32P incorporation, as did the later lag phases and logarithmic phase [Fig. 7, lane 5-12). Incorporation of 32P into the DNAs of the cell suspension showed that there was temporal synthesis of DNAs When replication was renewed from quiescent cells in stationary phase the 1.9 kb minicircle and chloroplast DNA first began replication followed by replication of the nuclear and principal mitochondrial genomes. The early synthesis of the 1.9 kb minicircle led to an increase in its copy number relative to that of the other small DNAs. As the growth continues in the new medium the copy number and amount of 32P incorporation into the small DNAs resembled that of cells in logarithmic phase.

Distribution of the 1.9 and 1.4 kb Minicircular DNAs

Mitochondrial DNA isolated from a number of maize cytoplasms was examined for the presence of the 1.4 and 1.9 kb minicircular DNAs. Mitochondrial DNA was prepared from Black Mexican cell suspension (N, WF9(N, WF9 T), and WF9(C), WF9(S) and MB25(vg, cytoplasms and the corresponding spontaneous cytoplasmic revertents to fertility of the WF9(S) and MB25(vg, cytoplasms. The full length 1.4 kb clone and the 1,752 bp Bam HI clone of the 1.9 kb minicircular DNA were nick translated and used as probes on Southern blots of the above mitochondrial DNAs. The 1.9 kb minicircular DNA was present in all the cytoplasms except vg and the vg revertant, the 1.4 kb minicircular DNA was present in only the C and the cell suspension cytoplasms (Fig. 8).

In a survey of twelve members of the S group of male-sterile cytoplasms, the 1.9 kb minicircle was found in three out of the twelve cytoplasms while the 1.4 kb minicircle was present in only one of the 5 cytoplasms (Fig. 9). The intense pair of bands above the 2.3 kb minilinear DNA were the S1 and S2 minilinear DNAs (Fig. 9). The heavy bands in Fig. 9, lane 5 are the cDN-double stranded RNAs, other bands between the S1 and S2 molecules and the 2.3 kb molecule were degradation products of S1 and S2. A prolonged exposure of the S cytoplasm blot with the probe of the 1.4 kb minicircle allowed the detection of a very small amount of the 1.4 kb minicircular DNA in five additional S cytoplasms (Fig. 10). The longer exposure also detected a small amount of hybridization of the 1.4 kb minicircle to the 1.9 kb minicircle (Fig. 10, lane 2).

Five C cytoplasms, each in two different nuclear backgrounds, were probed with 1.9 and 1.4 kb DNAs. The 1.9 kb DNA was present in all C

cytoplasms tested regardless of the two nuclear backgrounds, the 1.4 kb DNA was present in all the C cytoplasm except A619(Rb and A632 Rb, Fig. 11). Neither minicircle was present in all maize cytoplasms, the 1.4 kb minicircle was present in high copy number in only those cytoplasms which carried the 1.9 kb minicircle. Multimers with up to three or more repeating units of the minicircles were detected. When digested with a restriction endonuclease that has only one recognition site in the molecule, the multimers migrated as a single linear band (Fig 9 and 10 panel C). Thus, the multimers were head to tail multimers repeating unit.

It has been demonstrated that maize mitochondrial DNA sequences can be detected in the nuclear genome (Kemble et al. 1983). To test the possibility that those cytoplasms which lack the 1.9 and 1.4 kb sequences in their mitochondrial genomes had these sequences sequestered in their nuclear genomes, the 1.9 and 1.4 kb DNAs were hybridized with selected nuclear genomes. Nuclear DNA lines in S cytoplasm characterized by mitochondria with undetectable 1.4 and 1.9 kb DNA was prepared and hybridized with cloned 1.4 and 1.9 kb DNAs. One of the seven nuclear genomes (a Funk's Seeds International proprietary line, tested contained sequences homologous to both of the minicircular DNAs. The homology to the 1.9 kb DNA was integrated in Bam HI fragments of 8.0 and 4.8 kb. The homology to the 1.4 kb DNA was integrated into Bam HI fragments of 8.7, 7.8 and 4.2 kb (Fig. 12). No free 1.9 or 1.4 kb minicircles were present in any of the tested S cytoplasm nuclear genomes.

Expression of the Minicircular DNAs

Total mitochondrial RNA was denatured, electrophoresed, blotted to nitrocellulose and hybridized with strand specific probes. The probes were both strands of the full length 1.4 kb minicircle or the 1,752 bp Bam HI fragment of the 1.9 kb minicircle cloned into M13mp8. The 1.9 and 1.4 kb (+) strands hybridized to RNAs of 1,000 and 600 nucleotides, and 890, 700 and 460 nucleotides in length, respectively (Fig 13, lanes 2 and 5). The 1.9 kb (-) strand hybridized very lightly to an RNA which was 900 nucleotides in length (Fig 13 lane 6). The 1.4 kb - strand did not hybridize to any RNA (Fig. 13 lane 8). All of the RNA preparations were contaminated with minicircular DNA, even though the RNA was pelleted through a CsCl solution which should have eliminated the DNAs. When the mitochondrial RNA preparations were RNAse treated prior to denaturation and electrophoresis the RNA bands described above did not appear and the bands which were full length minicircular DNA remained.

The intactness of the RNAs was determined by staining the RNA with ethidium bromide to visualize rRNA and by hybridization with maize rDNA cosmid clones 2c13 and 2c21 (Stern, Dyer and Longdale 1982). The RNAs were judged intact if they did not contain bands other than the mitochondrial and cytoplasmic rRNAs Fig. 13, lanes 7 and 8,. The relative positioning of the multiple transcripts from each minicircle was not determined.

Nucleotide Sequence of the 1.4 kb Minicircle

The complete nucleotide sequence of the 1.4 kb minicircle was determined using the M13 clones produced by truncating both orientations of full length 1.4 kb M13 clones (Fig. 14 . The sequence of the (+) strand totals 1,445 bp (Fig. 15). Nucleotide one was assigned to the unique Eco RI site. The overall G+C content was 44.74%; three areas of low G+C content were uncovered when the % G+C was determined in 20 bp units and plotted against the length of the minicircle (Fig. 15 . The low G+C areas were between nucleotides 761-792, 937-971 and 1,381 1413, and are 19.35%, 14.71% and 25.00% G+C, respectively. The low G+C area between 937-971 bp contained a direct repeat of a 12 bp sequence (ATTTATAATTAG) with only three mismatches. The repeating unit was separated by 2 bp.

The 1.4 kb sequence had a perfect direct repeat which is located between 1197-1239 bp. The repeating unit was 18 bp long, separated by 5 bp. Other direct repeats of up to 13 bp existed in the 1.4 kb minicircular DNA (Fig. 15) The molecule also had several areas which have the potential to form hairpin or stem loop structures.

Numerous small open reading frames were detected on the 1.4 kb sequence. The three longest open reading frames which were detected on the +, strand were located between nucleotides 120-350, 1240-1353, and 1245-1400, these open reading frames (designated 1, 2, and 3, respectively, would encode for proteins which are 77, 38, and 58 amino acids long, respectively. The TGA codon is a stop codon in the nuclear triplet code or the amino acid tryptophan in the mitochondrial triplet code for animals and fungi. However, the apocytochrome b gene of Oenothera stops with the TGA codon (Schuster and Brennicke 1985,.

Therefore the search for open reading frames was performed using TGA as mitochondrial stop codon. The open reading frame from 120-350 stops with the TGA codon while the other two open reading frames stop with TAG.

Sequences which may act as ribosome binding sites were located 5' to the open reading frames. These sequences have the ability to hybridize with the 18S rRNA sequence 3' ...UCCUAAGU...5' (Dawson, Jones and Leaver 1984). These octanucleotide sequences end -23, -9, and -14 bp away from the AUG initiation codons of open reading frame 1, 2, and 3, respectively. Each ribosome binding site has six out of eight nucleotides capable of hybridizing with the sequence of the 18S rRNA. Sequences of the ribosome binding sites of some maize mitochondrial genes were not conserved in the corresponding wheat mitochondrial genes (Boer et al. 1985). The significance of the presence of a ribosome binding site in the minicircle open reading frames is unknown.

The sequence of 1.9 kb minicircle has been completed (Ludwig et al. in preparation) and was compared to the sequence of the 1.4 kb minicircle. The 1.4 kb minicircle has 62 bp of near perfect homology with the 1.9 kb minicircular DNA. There are 3 mismatches in the 62 bp sequence without looping out either sequence to continue the match. The sequence is located between nucleotides 1300 and 1361. This consistant with the hybridization of the 1.4 kb probe to the 1.9 kb minicircle (Fig. 1D). When the homologous 62 base pairs were aligned no other areas of significant homology were found. No other areas of significant homology were found between the 1.4 kb minicircle and the other areas of the 1.9 kb minicircle, the 5 cytoplasm molecule 5S (Levings and Sederoff

1983 , a DNA minicircle from male-fertile sugarbeet (Hansen and Marcker 1984) or cytochrome oxidase subunit II of maize (Fox and Leaver 1981 ,

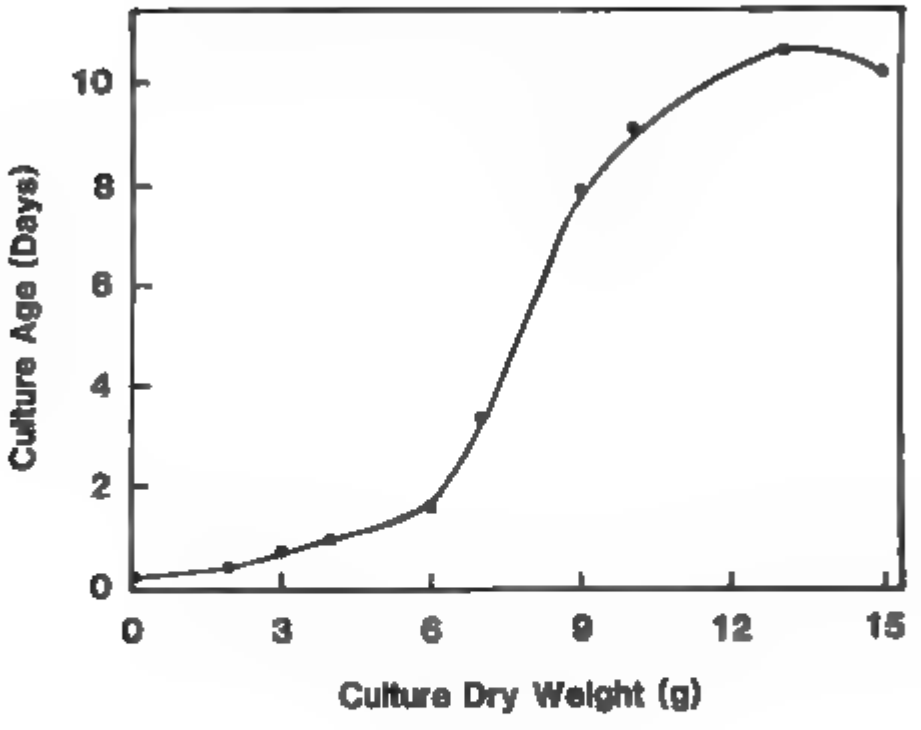


Fig. 1. Growth curve of the Black Mexican cell suspension. 50 ml cultures were inoculated with 5 ml of logarithmic growing cultures. Two cultures were harvested at various times by filtration and oven dried at 80 C with vacuum to a constant weight.

A

B

Fig. 2. Photomicrographs of cells from a logarithmic phase (A) and stationary phase (B) cell suspension of Black Mexican maize. Tested for viability by staining with an equal volume of 0.005% w/v fluorescien diacetate.

fig. 3. Agarose gel electrophoretic patterns of Black Mexican cell suspension mitochondrial DNA digested with exonuclease III (1) and undigested (2). Arabic numerals refer to the linear size of the DNA in kb. The roman numerals refer to conformation of the DNA: I, linear; II, open circle; and III, covalently closed circle.

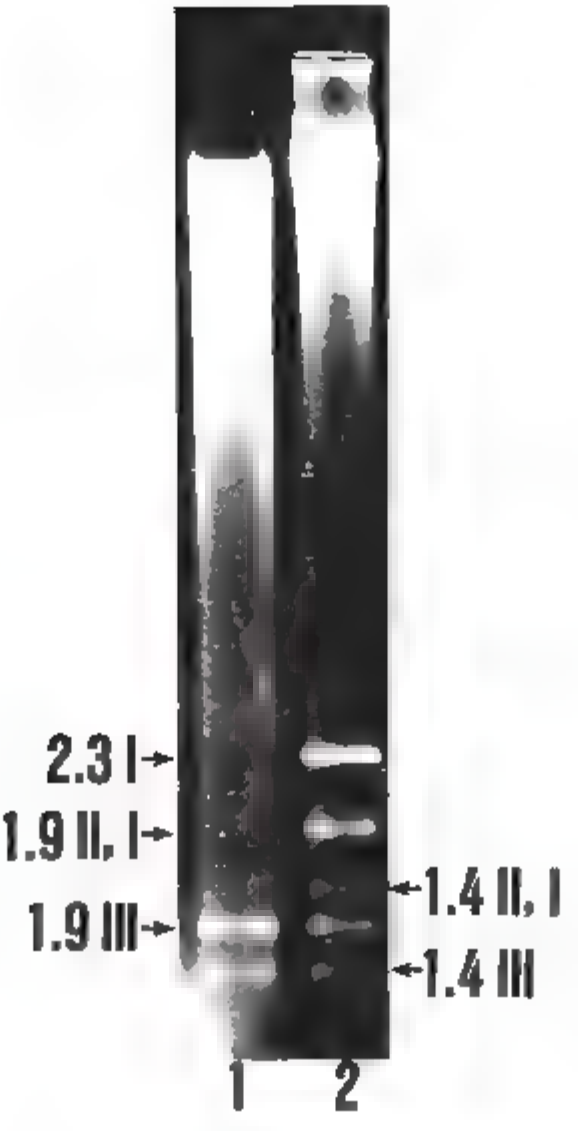
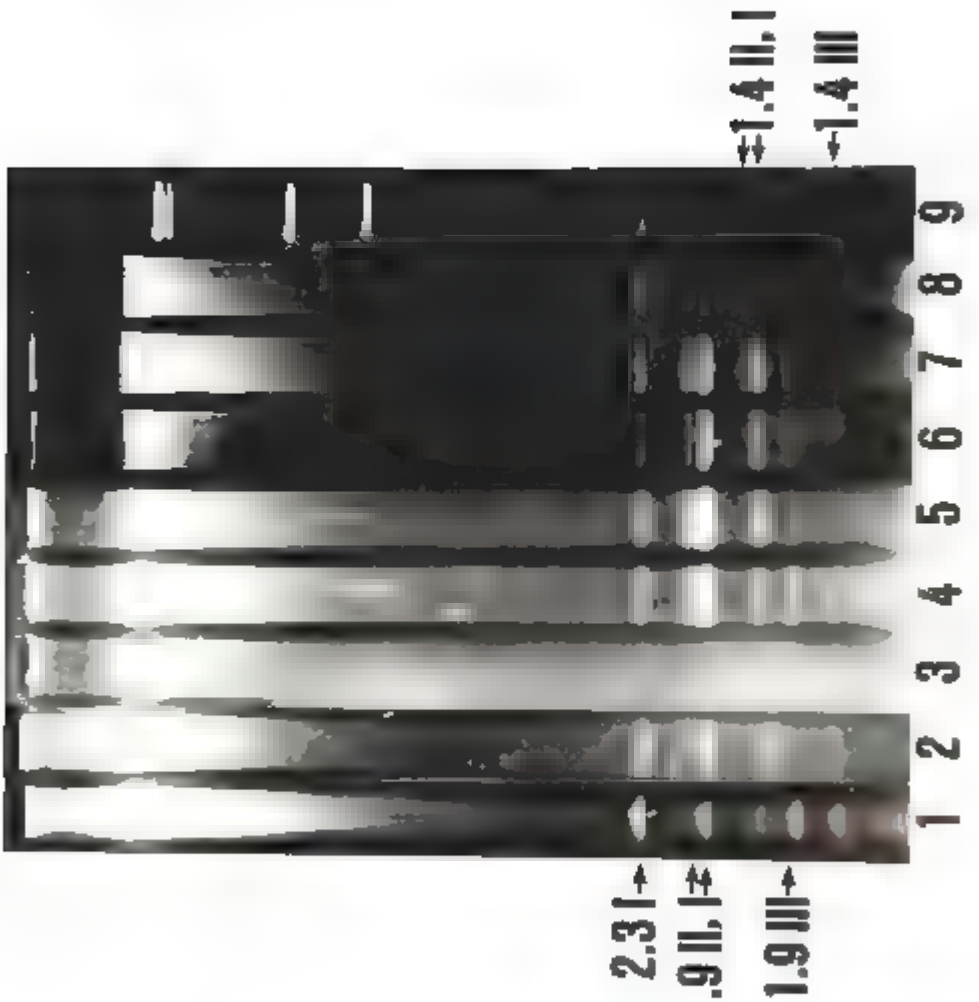

2.3 I
1.9 II, I
1.9 III
1.4 II, I
1.4 III
1
2

Fig. 4. Agarose gel electrophoretic patterns of mitochondrial DNA isolated from leaves of green plants (1) and various growth phases of the Black Mexican cell suspension (2-8). Cell suspension DNA was isolated from cultures in logarithmic phase (2), stationary phase (3), one day in lag phase (4), two days in lag phase (5), three days in lag phase (6), five days in lag phase (7), and seven days in lag phase (8). Lane 9 is lambda DNA digested with *Hin* dIII. Arabic numerals refer to the linear size of the DNA in kb. The roman numerals refer to conformation of the DNA: I, linear; II, open circle; and III, covalently closed circle.


1 2 3 4 5 6 7 8 9
2,3 I
1,9 II, I
1,9 III
1,4 II, I
1,4 III

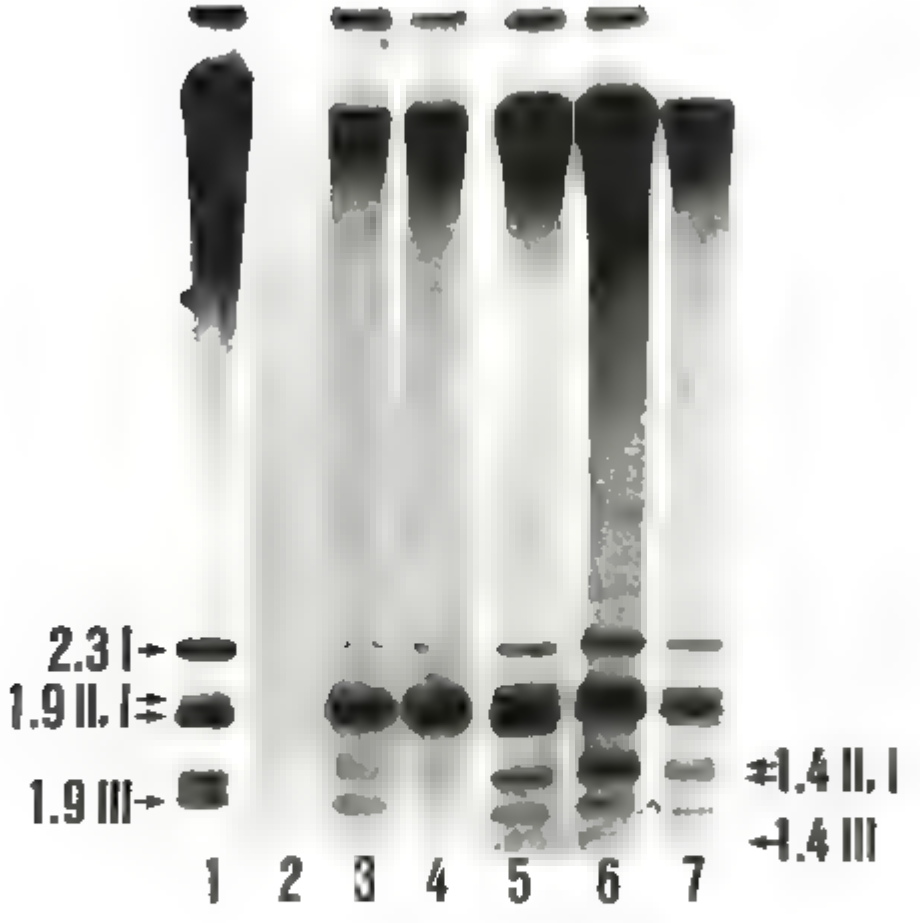


Fig. 5. Autoradiogram of lanes 2-8 of the agarose gel shown in Fig. 4. Cultures were incubated with 15 microcuries per milliliter of phosphorous 32, 24 hr prior to isolation of DNA. Black Mexican cell suspension DNA was isolated from cultures in logarithmic phase (1), stationary phase (2), one day in lag phase (3 , two days in lag phase (4), three days in lag phase (5), five days in lag phase (6), and seven days in lag phase (7). Arabic numerals refer to the linear size of the DNA in kb. The roman numerals refer to conformation of the DNA: I, linear, II, open circle; and III, covalently closed circle.

Fig. 6. Ethidium stained agarose gel electrophoretic pattern (1, 2, 4, 6, 8, 10, 12) and corresponding autoradiograms (3, 5, 7, 9, 11, 13) of chloroplast (1) and Black Mexican cell suspension mitochondrial DNA (2-13) digested with Bam HI restriction endonuclease. Cell suspensions were incubated with 15 microcuries per milliliter of phosphorous 32, 24 hr prior to isolation of mitochondrial DNA. Cell suspension DNA was isolated from cultures in stationary phase (2 and 3), one day in lag phase (4 and 5), two days in lag phase (6 and 7), five days in lag phase (8 and 9), and seven days in lag phase (10 and 11) and logarithmic phase (12 and 13). The arrow indicates the position of the 1.752 kb Bam HI fragment of the 1.9 kb minicircle.

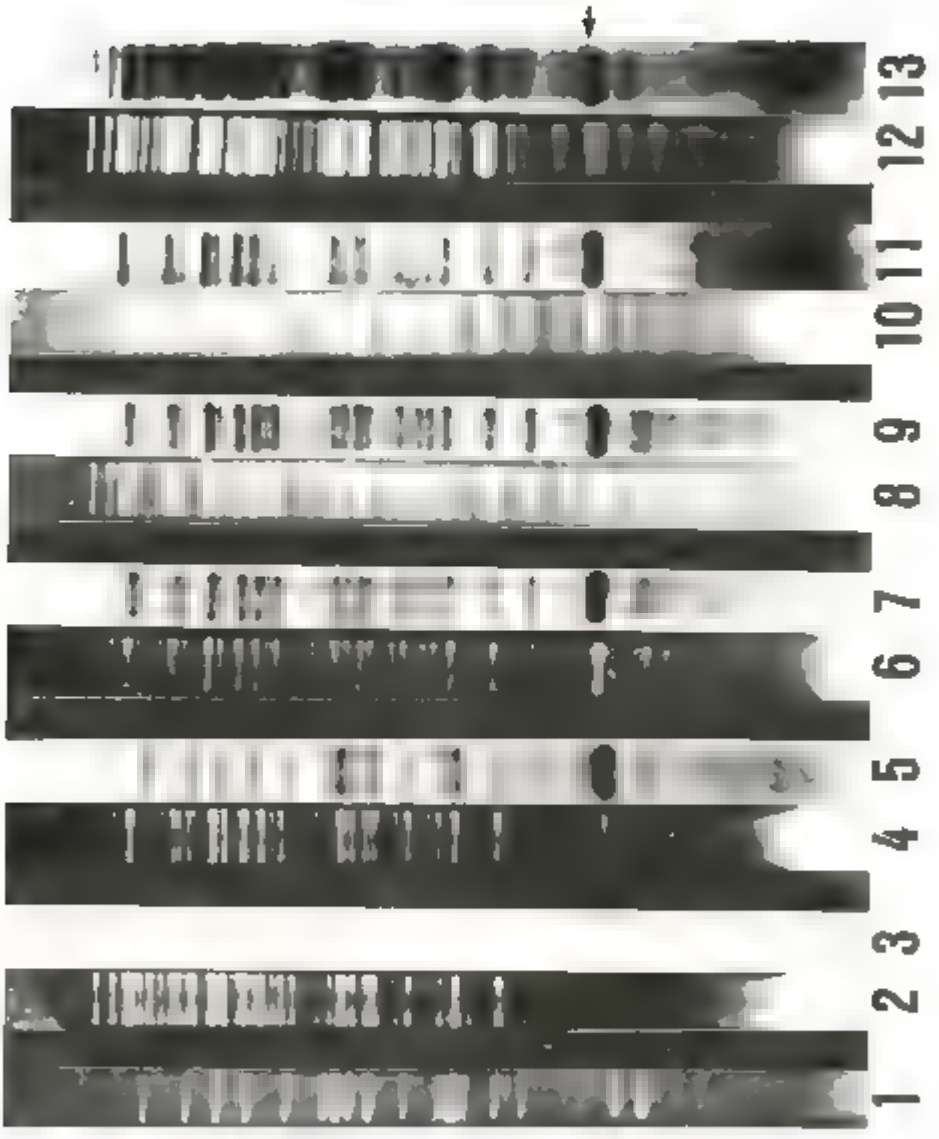

1
2
3
4
5
6
7
8
9
10
11
12
13

Fig. 7. Ethidium stained agarose gel electrophoretic patterns (odd numbered lanes) and corresponding autoradiograms (even numbered lanes) of Black Mexican cell suspension nuclear DNA digested with Bam HI restriction endonuclease. Cell suspensions were incubated with 15 microcuries per milliliter of phosphorous 32, 24 hr prior to isolation of mitochondrial DNA. Cell suspension DNA was isolated from cultures in stationary phase (1 and 2), one day in lag phase (3 and 4 , two days in log phase (5 and 6), five days in lag phase (7 and 8), seven days in lag phase (9 and 10) and logarithmic phase (11 and 12).

Fig. 8. Ethidium bromide stained (B) and hybridization of the 1.752 kb Bam HI clone of the 1.9 kb minicircle (A) and full length clone of the 1.4 kb minicircle (C) to mitochondrial DNA. Mitochondrial DNA was isolated from maize lines WF9(N) (1), WF9(T) (2), WF9(C) (3), WF9(S) (4), a fertile mutant of WF9(S) (5), M825(Vg) (6), a fertile mutant of M825(Vg) (7), Black Mexican cell suspension (8), and twice the amount of DNA as the adjacent lane from Black Mexican cell suspension (9). Arabic numerals refer to the linear size of the DNA in kb. The roman numerals refer to conformation of the DNA: I, linear; II, open circle; and III, covalently closed circle.

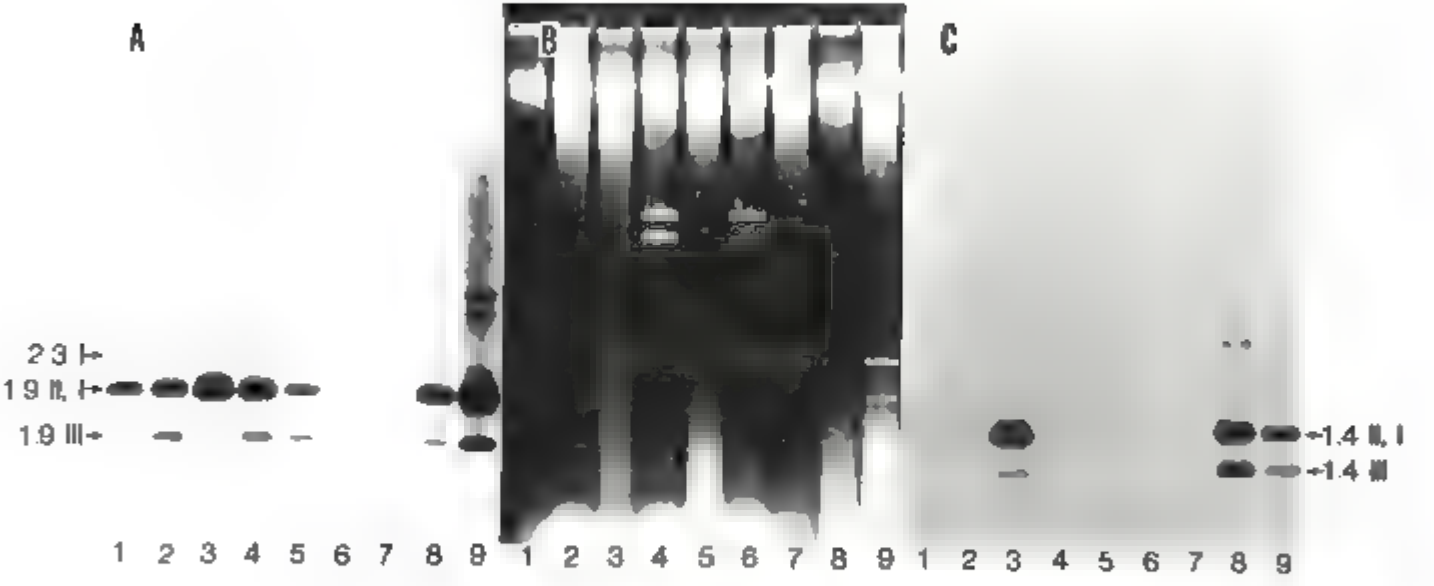

A
B
C
23 I
19 II, I
19 III
1.4 II, I
1.4 III
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9

Fig. 9. Ethidium bromide stained gel (B), hybridization of the 1.752 kb Bam HI clone of the 1.9 kb minicircle (A) and clone of the 1.4 kb minicircle (C) to mitochondrial DNA. Mitochondrial DNA was undigested (1-13) and digested with either Bam HI (A, 14) or Eco RI (B and C, 14). Mitochondrial DNA was isolated from maize lines W182BN(D) (1), W182BN(S) (2), W182BN(ME) (3), W182BN(CA) (4), W182BN(LBN) (5), W182BN(B) (6), Funk's Seeds International proprietary line (R) (7), NS(S) (8), 3811(Ms) (9), NS(Ms) (10), LERF(Ms) (11), 3811(S) (12), Black Mexican cell suspension (13 and 14). Arabic numerals refer to the linear size of the DNA in kb. The roman numerals refer to conformation of the DNA: I, linear; II, open circle; and III, covalently closed circle.

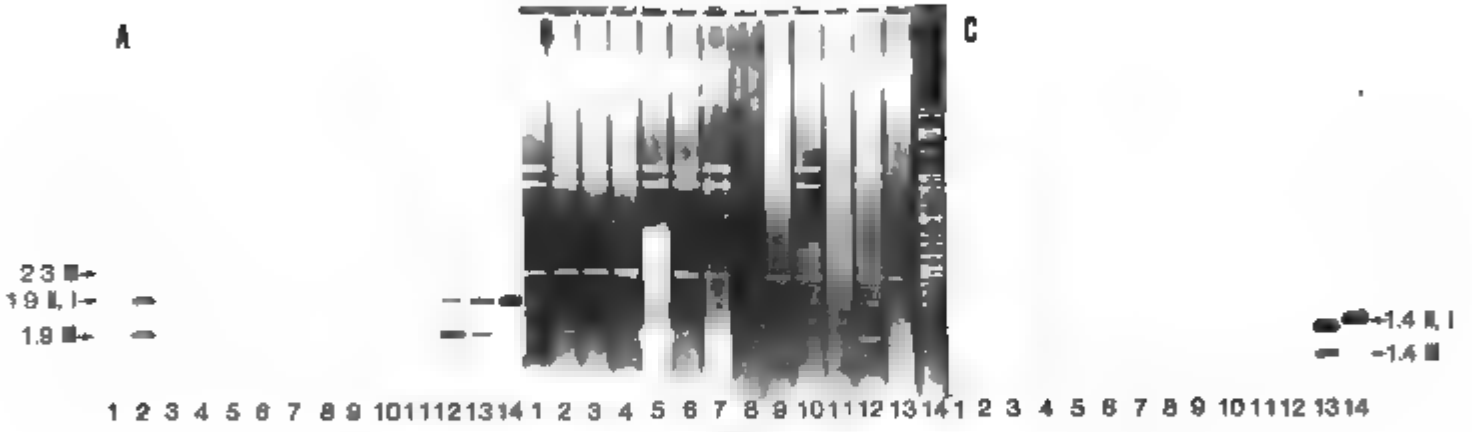
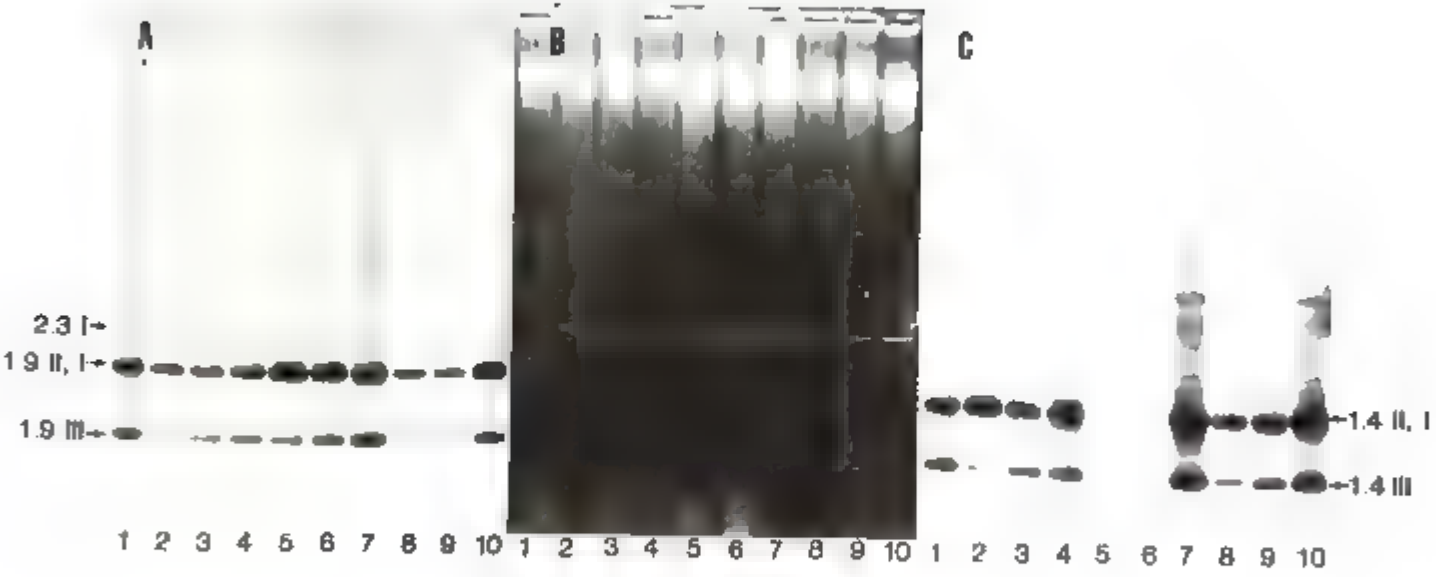

A
C
2.3 III
1.9 II, I
1.9 III
1.4 II, I
1.4 III
1 2 3 4 5 6 7 8 9 10 11 12 13 14 1 2 3 4 5 6 7 8 9 10 11 12 13 14 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 10. Prolonged exposure of autoradiogram C in Figure 9.


1.9 II, I
1.9 III
1.4 II, I
1.4 III
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 11. Ethidium bromide stained gel (B) and hybridization of the 1.752 kb Bam HI clone of the 1.9 kb minicircle (A) and full length clone of the 1.4 kb minicircle (C) to undigested mitochondrial DNA. Mitocondrial DNA was isolated from maize lines A619 (odd numbered lanes) and A632 (even numbered lanes) with cytoplasms BB (1 and 2), Pr (3 and 4), Rb (5 and 6), ES (7 and 8), and C (9 and 10). Arabic numerals refer to the linear size of the DNA in kb. The roman numerals refer to conformation of the DNA: I, linear; II, open circle; and III, covalently closed circle.


A
B
C
2.3 I
1.9 II, I
1.9 III
1.4 II, I
1.4 III
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10

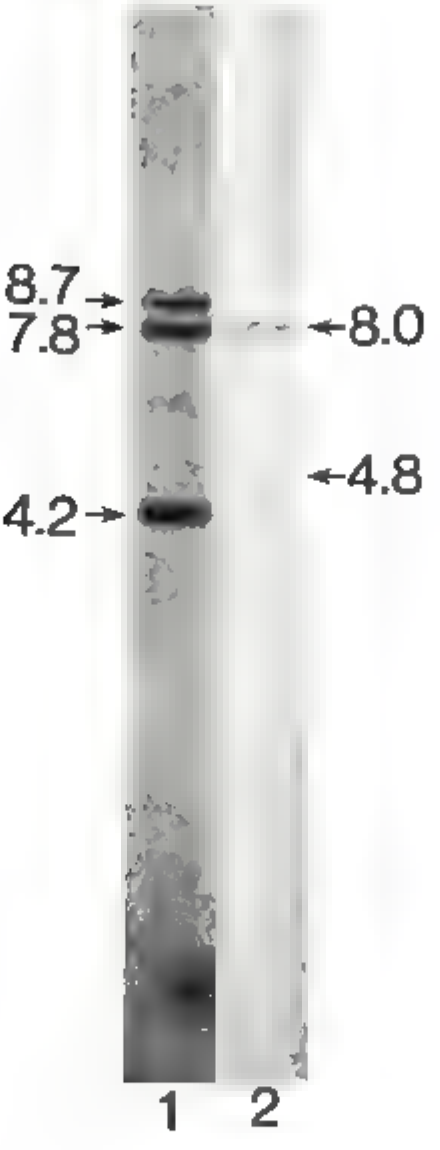
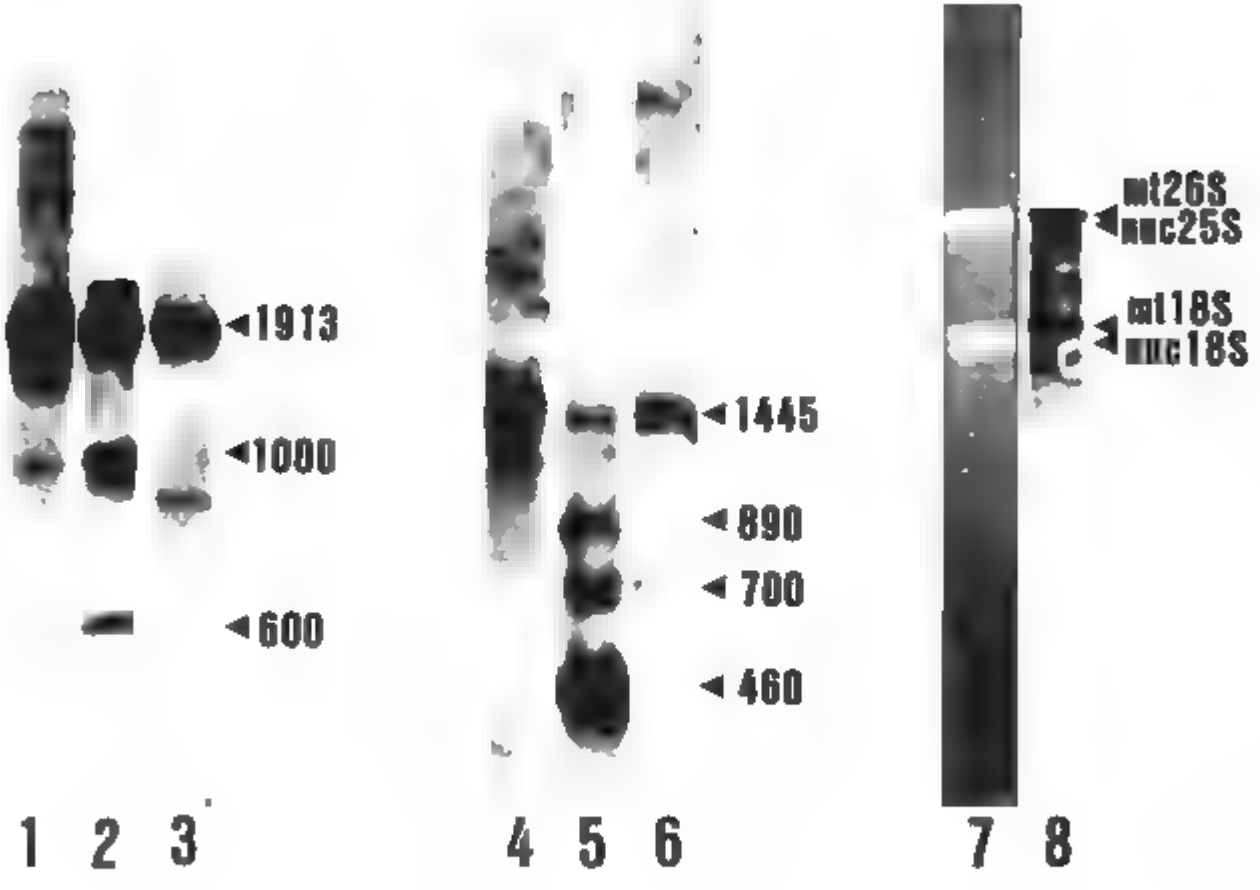


Fig. 12. Hybridization of the full length clone of the 1.4 kb minicircle (1) and the 1.752 kb Bam HI clone of the 1.9 kb minicircle (2) to maize nuclear DNA isolated from the Funk's Seeds International proprietary line (P). 15 micrograms of Bam HI restriction endonuclease digested DNA was loaded in each lane. Arabic numerals refer to the linear size of the DNA in kb.

Fig. 13. Hybridization of the the 1.752 kb Bam HI M13 (+) (1 and 2) and (-) (3) clones of the 1.9 kb miricircle and full length M13 (+) (4 and 5) and (-) (6, clones of the 1.4 kb miricircle to mitochondrial DNA (1 and 4) and RNA (2, 3, 5, 6, 7 and 8). Intactness of RNA was determined by ethidium bromide staining of the glyoxyl gels (7) and hybridization with the mitochondrial-ribosomal DNA clones 2C13 and 2C21 (8) (Stern, Dyer and Lonsdale 1982). 10 micrograms of RNA and approximately 0.01 micrograms of DNA were glyoxylated, electrophoresed and transfered to nitrocellulose (Thomas 1983). 5 micrograms of RNA was electrophoresed in lanes 7 and 8.


1913
1000
600
1445
890
700
460
mt26S
nuc25S
mt18S
nuc18S
1 2 3
4 5 6
7 8

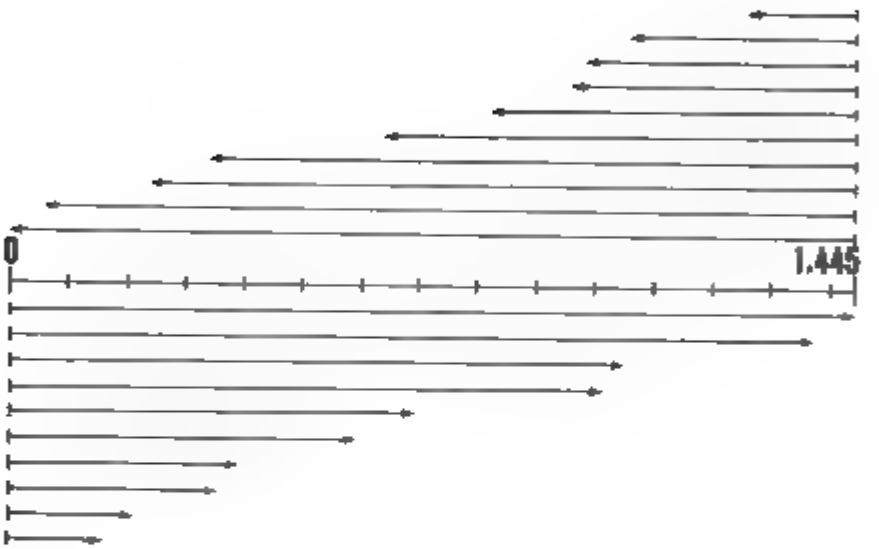
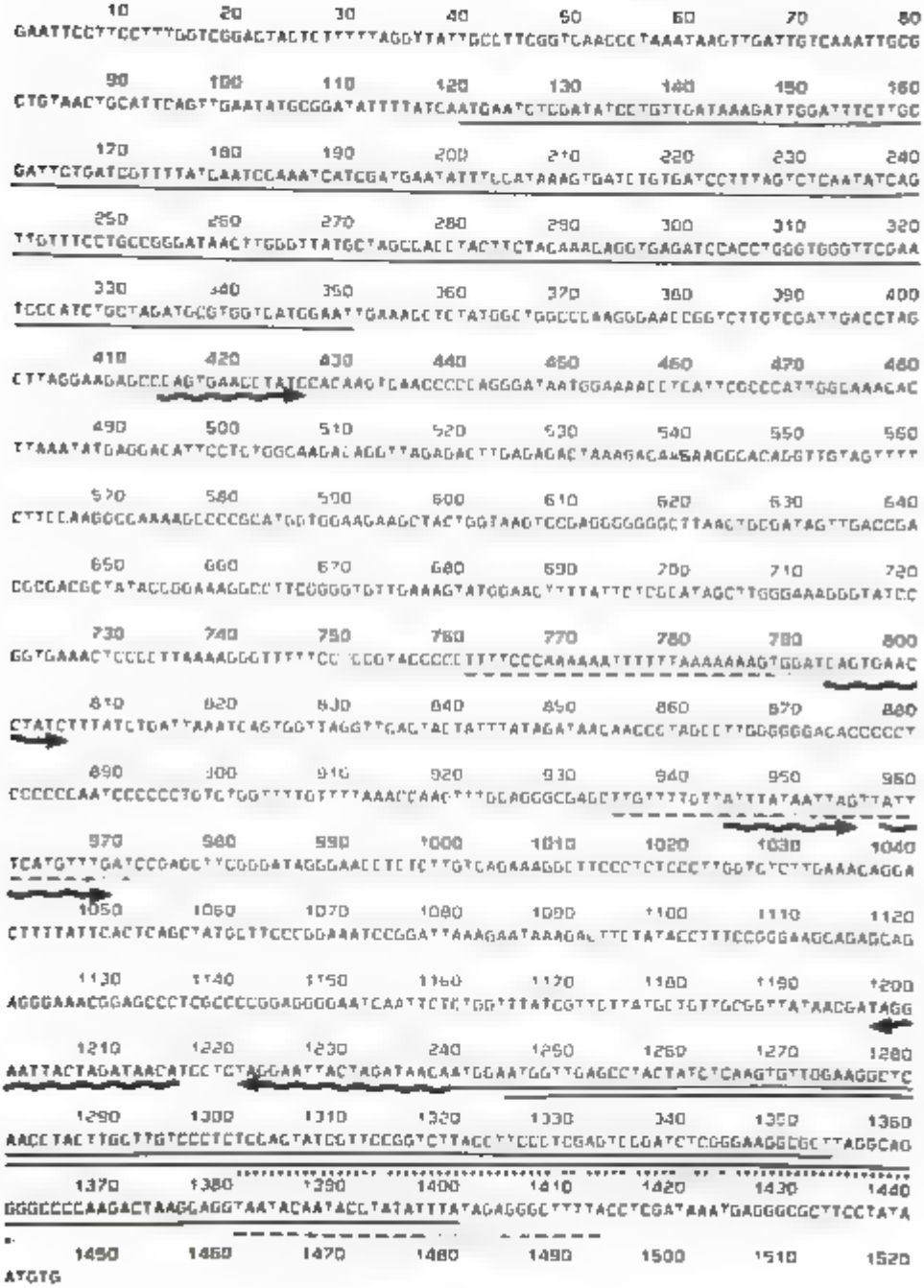


Fig. 14. Map of M13 clones (+) strand (upper) and (-) strand (lower) used to sequence the 1.4 kb minicircular DNA. Replicative form of both orientations of the 1.4 kb fragment cloned into the Eco RI site of M13mp8 were digested with with Bam1 HI and Pst I restriction endonucleases. The DNAs were then truncated with an exonuclease III and VII treatment, made blunt ended, ligated and transformed into JM109 (Yanish-Perron, viera and Messing 1984). The arrows point in the direction that the clones were truncated from. The primer annealing site is now located just to the left of the arrows for the (+) strand and just to the right of the arrows for the (-) strand. Sequence reactions are polymerized in the opposite direction of the arrows.

Fig. 15. Sequence of the 1.4 kb minicircle (+) strand. Solid lines locate open reading frames, waved lines locate direct repeats, dashed lines locate regions of low G+C content and the dotted line indicates the 82 bp region which is homologue to the 1.9 kb minicircle.



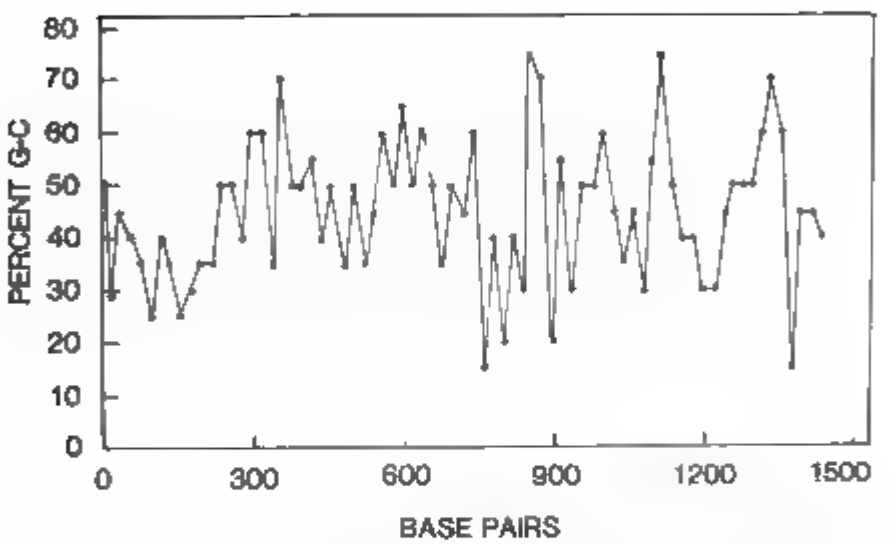


Fig. 16. Content of G+C in the 1.4 kb minicircular DNA. Percent G+C was calculated in 20 bp regions from bp 0 through 1445 (Fig. 14).

DISCUSSION

DNA Synthesis in Cell Suspension Cultures

The mitochondrial genome of Black Mexican maize consists of a high molecular weight principal genome and a group of minilinear and minicircular DNAs. The smaller DNAs consisted of a 2.3 kb minilinear DNA, the 1.4 and the 1.9 kb minicircular DNAs. The principal genome was visualized as a smear at the top of agarose gels, and the smaller DNAs could be distinguished as discrete bands in the same preparations. The 2.3 kb minilinear DNA electrophoresed as a single linear form, whereas the 1.4 and 1.9 kb DNAs electrophoresed as a linear, open circular and covalently closed circular forms. Head to tail multimers of both minicircular DNAs were detected by Southern hybridization. These multimers are most likely formed during replication of the minicircles, but may be formed by intermolecular recombination. The components of the mitochondrial genome were the same in DNA extracts from Black Mexican cell suspension or from plant.

The cell suspension used to study mitochondrial DNA synthesis has several growth phases. These include lag, logarithimic and a stationary growth phases. The cells in stationary phase were viable and could be used as inoculum for continued growth but were quiescent in that no detectable DNA synthesis occurred over a 24 hr period and the cells had a decreased fluorescence when stained with fluorescien diacetate.

Although the lag phase cultures may or may not reflect the *in vivo* conditions of the plant, the lack of DNA synthesis and the ability to renew DNA synthesis made the quiescent cells valuable for the study of DNA replication.

DNA synthesis was not detected in stationary phase cell suspensions. Stationary phase cells began synthesis of DNA after being placed into new medium. The renewed DNA synthesis was first detected in proplastid, minicircular and minilinear DNAs, followed by synthesis of the principal mitochondrial genome and nuclear DNA. There was a temporal sequence of DNA synthesis when quiescent cells renewed synthesis of DNA. This may indicate that synthesis of maize DNAs occurs in an ordered fashion or that those DNAs showing earliest incorporation were much more efficient replicons.

An analogous phenomenon has been described in anerobically grown yeast cultures when the cells are exposed to oxygen (Rabinowitz et al. 1969). As mitochondrial function developed in the aerobic cultures, incorporation of radioactivity into mitochondrial DNA was 6 to 30 times greater than incorporation into nuclear DNA. Little mitochondrial synthesis is occurring in the anaerobically grown yeast cultures. As mitochondria adapt to the aerobic condition and begin to function, mitochondrial replication begins either much faster or prior to nuclear DNA synthesis.

The quiescent cells renew synthesis of DNA first in the 1.9 kb minilinear DNA of the mitochondria and in the proplastid DNA. The proplastids of the cell suspension never fully differentiate into chloroplasts. After these DNAs have incorporated label the principal mitochondrial genome and the nuclear genome begin DNA replication. As

in the yeast cultures, this may represent the early adaptation of the proplastids prior to the mitochondria. Cells may require some proplastid function, which indirectly requires proplastid division and the replication of its genome. Mitochondrial DNA replication may not be needed for the adaptation to the new medium. The 1.9 kb minicircular replication could be necessary for replication or may be a much more efficient replicon relative to the principal genome.

Using various inhibitors of cytoplasmic or organelle protein and DNA synthesis, Heinhorst, Gordon and Weissbach (1985) found that inhibition of chloroplast DNA synthesis in suspension cultures of N. tabacum had little effect on replication of nuclear DNA and that inhibition of nuclear DNA synthesis had an indirect or delayed inhibition of chloroplast DNA synthesis. From these data they concluded that plastid and nuclear DNA synthesis were not coupled in N. tabacum. This is in agreement with the unlinked synthesis of nuclear and mitochondrial DNA in vertebrates and yeast (Bogenhagen and Clayton 1977 Blanc and Dujon 1982). Synthesis of DNA occurred in an ordered fashion in maize cultures which were renewing DNA replication. The temporal sequence of DNA synthesis seen in lag cultures of the maize cell suspensions may be a result of adaptation to the environmental conditions and may not be a reflection the intrinsic order of DNA synthesis in the plant.

The two minicircular DNAs had increased copy numbers in the cell suspension relative to plants. The environment of the tissue culture as opposed to the plant results in a greater replication of the minicircular DNAs relative to the minilinear DNA. This could be due to genetic changes, either nuclear or cytoplasmic, which have occurred

since the original explants were placed into culture. The change could have occurred in the mechanism which controls copy number or the system which replicates the small DNAs. The change could be epigenetic which makes the prediction that if plants were regenerated from the cell suspension the original plant copy number would be reestablished.

Distribution of the Maize Minicircular DNAs

The amplified and early synthesis of the 1.9 kb minicircular DNA can be correlated with other properties of this DNA relative to the 1.4 kb minicircular DNA. The early synthesis and amplification of the 1.9 kb minicircle may be a factor related to the higher copy number reached by this DNA relative to the 1.4 kb minicircle. The higher copy number could in turn increase the frequency of transmission of the 1.9 kb minicircle when mitochondria and cells divide. This suggests that the 1.4 kb DNA would be lost more often than the 1.9 kb DNA due to random segregation. The relationship of replication efficiency and copy number to frequency of transmission of a mitochondrial replicon has been studied in hypersuppressive strains of yeast. Suppressiveness of the petite mutant towards the wild type genome is thought to be due to the preferential replication of the petite genome relative to the wild type genome (Blanc and Dujon 1980). As the relative copy number of the petite genome increases, the wild type genome is lost. When mitochondrial markers are followed during yeast matings and subsequent heterocytoplasm formation, correlations have been found between the input frequency of an allele and the frequency of the allele in the zygote population (reviewed by Birky 1983). The higher copy number of the 1.9 kb minicircle would predict an increased frequency of

transmission during mitochondria division and DNA segregation relative to the 1.4 kb minicircle.

Distribution of the two minicircles in maize cytoplasms corroborated the prediction that the 1.4 kb minicircle could have been lost more frequently than the 1.9 kb minicircle. The 1.4 kb minicircle was found in fewer maize cytoplasms than the 1.9 kb minicircle (Pring and Smith 1985 . Other explanations can be made for the reduced presence of the 1.4 kb minicircle. The 1.4 kb minicircle may have never been present in these cytoplasms. The minicircular DNAs may have other factors such as their primary sequence or gene products which affect their transmission efficiency and stability with the cell. Nuclear and mitochondrial interactions may be the direct reason for the relative copy number of the small DNAs and their distributions within maize cytoplasms. Nuclear genotype can alter the copy number of the S1 and S2 DNA (Laughnan, Gabay-Laughnan and Carlson 1981). These data do not rule out the possibility of the gain or loss of the small DNAs being completely random. However, it is attractive to explain this phenomenon by correlating the known biological properties of early synthesis and higher copy number of the 1.9 kb minicircle relative to the 1.4 kb minicircle.

The relative copy number of the minicircles varies between plant and cell suspension and among the culture growth phases, but there also appears to be a stable control on the copy number of the minicircles. The DNA isolated from logarithmic cultures over a four year period showed no noticeable differences in copy number or composition of the small DNAs, whereas changes in the principal mitochondrial genome after culturing of plant cells has been documented in maize and Nicotiana

(McNay, Chourey and Pring 1984, Gengenbach et al. 1981, Kemble, Flavell and Brettel 1982, Dale, Wu and Kiernan 1983). When clones of the 1.9 kb minicircle from Black Mexican and B79 lines of maize were compared only three bp changes were noted in the 842 bp, which were sequenced (Ludwig et al. in preparation). In the survey of maize cytoplasms no homologous sequences to either minicircle were found in any other maize DNA of other than the two minicircular DNAs. The minicircles may lack the ability to transpose or recombine with any other of the mitochondrial DNAs or between themselves in maize.

The lack of sequence homology between minicircular DNAs and the principal mitochondrial genome has been noted with most of the higher plant cytoplasms examined The Neurospora mitochondrial minicircles which are not suppressive towards the principal genome also share no homology with the principal genome. This property among minicircular DNAs may indicate a non-mitochondrial origin of these minicircles. The survival or growth rate of a plant would be low in the presence of a suppressive minicircular DNA such as those found in the petite mutants of yeast because of the obligate requirement for functional mitochondria of the higher plant. Higher plant mitochondrial DNA minicircles, without homology to the principal genome may be retained because they are not suppressive of the principal genome. Lack of homology to the principal genome could reduce competition as a replicon and or recombination with the principal genome. Abbott, O'Dell and Flavell (1985) detected sequences homologous to the 1.9 kb minicircle in the principal mitochondrial genome of some maize lines. All cytoplasms surveyed in this study for the presence of sequences homologous to the 1.4 and 1.9 kb DNAs were also surveyed after digestion with Bam HI or

Eco RI. No homologous principal genome sequences were detected. This may be due to differences in hybridization conditions.

In the cytoplasms surveyed in this study no cytoplasm was found which contained the 1.4 minicircular DNA without the 1.9 kb minicircle. These data may indicate a dependence of the smaller circle on the larger for replication and or segregation to progeny mitochondria. The early replication of the 1.9 kb minicircle does indicate that the two small circular DNAs located within the mitochondria can be differentiated by the mitochondrial replication mechanism or that the two minicircles have different replication mechanisms. The replication of the 1.9 kb minicircle may be required prior to the replication of the 1.4 kb minicircle. This would explain the amplification and higher copy number of the 1.9 kb minicircle relative to the 1.4 kb minicircle.

The absence of both the 1.9 and 1.4 kb minicircular DNAs in some maize cytoplasms may indicate that these DNAs are dispensable to the maize mitochondria. The 1.9 kb DNA was found in all but one of 25 races of maize indigenous to Mexico surveyed (Kemble, Gunn and Flavell 1983). Carlson and Kemble [1985] divide the S type of male sterile cytoplasms by the presence or absence of the 1.9 kb minicircle. The 1.9 kb minicircle was found in 3 of the 14 S type cytoplasms examined. A conclusion from these surveys and the one performed in this study is that the 1.9 kb DNA is dispensable to the maize mitochondrion. However, dispensability can not be definitively concluded until a plant containing these minicircles is cured of all homologous sequences and shown to be equal to the progenitor containing the two minicircles. Until this experiment is performed it is still possible that these two minicircles are important in some manner to the cytoplasms in which they

are found. It should not be overlooked that these two minicircles or any of the small DNAs may in fact be detrimental to the plants where they are found.

Nuclear Homology to the Minicircular DNAs

Many of the proteins found in mitochondria are encoded in the nucleus, translated on cytoplasmic ribosomes and translocated into mitochondria (reviewed by Leaver and Gray 1982). The possibility that a function of the 1.9 and 1.4 kb minicircles could be nuclear encoded in those lines which did not contain the two DNAs in their mitochondria was tested by hybridizing nuclear DNA with 1.9 and 1.4 kb DNA sequences. Seven nuclear genotypes which did not contain 1.9 or 1.4 minicircle sequences in the mitochondria were examined. None of the nuclei contained free minicircular DNA. One of the seven genotypes contained integrated sequences which hybridized to both the 1.9 and 1.4 kb minicircular sequences. The complete lack of any 1.9 and 1.4 kb minicircular homologous sequences is an indication that these small DNAs are dispensable to the maize mitochondria and cell. Abbott, O'Dell and Flavell (1985) found sequences homologous to the 1.9 kb minicircle in the nuclear genome of M825 vg), an S type cytoplasm. The homologous sequences found in the nuclear genome may be the origin of these mitochondrial minicircles, vestigial sequences of a minicircular transposition, or random sequence homology.

Expression of the Minicircular DNAs

Transcripts homologous to the two small DNAs were detected. The 1.4 (+ strand hybridizes to three RNAs and has three open reading frames on the corresponding [+] strand. The 1.9 kb minicircle hybridizes to two RNAs on the (+) strand and one RNA on the [- strand and has a long open reading frame on the (+) strand [Ludwig et al. in preparation . The RNAs could be used directly as a primer for DNA synthesis or for other functions. However, the presence of open reading frames on the minicircles suggests that the RNAs may be translated. Each open reading has a sequence which may act as a ribosome binding site, however the exact requirements for mitochondrial gene expression in higher plants is unknown.

The presence of RNA molecules homologous to the minicircular DNAs indicates the DNAs may have a function in the mitochondria. The putative function and the minicircles may be indispensable to those mitochondria in which they are found. The presence of these minicircles may be correlated with positive, negative or neutral effects relative to survival of the maize plant.

Nucleotide Sequence of the 1.4 kb Minicircle

The primary sequence structure of the 1.4 kb minicircle was compared to that of the 1.9 kb minicircle [Ludwig et al. in preparation, and the sugar beet minicircle pO Hansen and Marcker 1984 . Each of the minicircles contains a large direct repeat which may be fortuitous or may be related to maintenance or the origin of the minicircles Although each of the minicircles contains a direct repeat the nucleotide

sequence of the individual repeats among the circles is not conserved. The presence of direct repeats within the master chromosome results in the production of smaller circles by recombination through the homologous repeats (Lonsdale, Hodge and Fauron 1984). No smaller circles homologous to either the 1.9 or the 1.4 minicircle have been found, indicating that intramolecular recombination is not occurring. The multimeric forms of the circles could be formed by intermolecular recombination which may or may not involve the direct repeats on the molecule.

A common feature between the two maize minicircles is the presence of a 12 bp sequence which is very homologous to the core sequence of autonomously replicating sequences (ARS) (Kearsey 1984). The conserved core sequence compiled by Kearsey 1984 is 5' A/T)TTTAT,Pu)TTT(A/T, 3'. The ARS element is repeated twice in the 1.4 kb minicircle and three times in the 1.9 kb DNA sequence (Ludwig et al. in preparation), both repeats are located in low G+C areas of the minicircles. The sequence of pO also contains a sequence which has 8 out of 11 bp in common with the conserved core sequence of the ARS element. The sequence is located between bases 440-449 and 490-499, which is located within the large direct repeat of pO (Hansen and Marcker 1984). The pO repeat does not appear to have a significantly less G+C content than the rest of the molecule.

Autonomously replicating sequences are largely composed of A+T rich sequences with a conserved core sequence (Kearsey 1984). Sequences which act as ARS elements have been isolated from wide variety of plant and animal species. There is evidence for ARS elements acting as *in vivo* nuclear origins of replication in yeast (Carriker and Campbell

1982) and Xenopus laevis (Zakian 1981). Sequences with ARS activity have also been isolated from the chloroplast genomes of Petunia hybrida (Overbeeke et al. 1985), tobacco (Ohmiya et al. 1983), and Chlamydomonas reinhardii (Vallet, Rahire and Rochaix 1984). Mitochondrial sequences which have ARS activity have been isolated from yeast (Hyman et al. 1982, Blanc and Dujon 1982) and Xenopus laevis (Zakian 1981). Most sequences with ARS activity have not been definitively associated with an in vivo origin of replication in the homologous organism.

Chloroplast sequences from C. reinhardii which are capable of supporting autonomous replication in the nucleus of C. reinhardii have been isolated (Rochaix, Dillewijn and Rahire 1984). These sequences are distinct from sequences with ARS activity and the in vivo origins of replication of the C. reinhardii chloroplast genome (Vallet, Rahire and Rochaix 1984, Vallet and Rochaix 1985). However, one of the two identified in vivo origins of replication, one of the eight ARS elements, and one of the four sequences which support autonomous replication in the C. reinhardii nucleus, are located within a single Eco RI restriction fragment. The interaction of the physically close elements during actual replication of the chloroplast genome is not determined. However, each of these activities can be physically separated on different restriction fragments (Vallet and Rochaix 1985).

It is unknown whether the sequences homologous to the core of the ARS element found in the three minicircular DNAs function as origins of replication for the minicircles. Each of the autonomously replicating minicircles must have at least one origin of replication. By comparing the three minicircular sequences it was possible to select a conserved

sequence which may act as an origin of replication. The high A+T region containing the sequences homologous to the yeast ARS element is the most likely candidate for an *in vivo* origin of replication.

The selection of the ARS like elements of the minicircles as origins of replication is substantiated by the known sequences which have been identified as yeast mitochondrial origins of replication. Mitochondrial origins of replication of yeast are consistently associated with high A+T content. The origin of replication consensus sequence compiled by Baldacci, Cherif-Zahar and Bernardi (1984) has a central 200 bp region of entirely A+T sequences. This is similar to the conserved rep sequence of yeast mitochondria which has long stretches of alternating AT pairs with both direct and indirect repeats (Blanc and Dujon 1980). It has also been shown that a mitochondrial replicon can consist entirely of A+T sequences only (Fangman and Dujon 1984). This supports the choice of the high A+T sequences of the 1.4 (this study) and the 1.9 Ludwig et al. in preparation kb minicircles as putative origins of replication. The most likely A+T rich regions are those which contain the ARS elements but the other high A+T regions may also be important in the replication of the minicircles.

The greatest conservation of sequence between the two maize minicircles is a 62 bp region with near perfect homology. This area is not significantly different in G+C content relative to the entire minicircles which makes it an unlikely origin of replication. The homologous region may indicate a common origin for the two molecules which has been conserved during their evolution. The sequences of the two molecules which do not share sequence homology could have evolved over time to their present divergence. The conserved area may import a

selective advantage for the molecules as they are replicated and or segregated.

The origin of most of the minicircles found in plants and fungi with the exception of those which are excised from the mitochondrial genome is unknown. Their presence in nearly all mitochondria which have been examined, indicates that their origins were not a rare event. Most minicircles, including the 1.4 and 1.9 kb DNAs do not have any detectable homology to the principal mitochondrial genome. Therefore they either had origins different than the principal genome or the homology between the principal genome and the minicircles has been lost. The presence of sequences homologous to the minicircles in the maize nucleus could be interpreted as the nucleus being the origin of the minicircular sequences. Conversely the nuclear homology could have resulted from a transposition of these sequences from the mitochondria either as integrated sequences prior to formation of autonomous minicircles or as autonomous minicircle sequences. These data presented do not preclude the possibility that the minicircular DNAs may have origins which are extracellular. Infection of a plant cell with a virus results in intracellular replication of a foreign nucleic acid within the plant cell. If a nucleic acid contained sequences which allowed stable replication within the mitochondria, a minilinear or minicircular molecule could be formed.

The presence of the minicircular DNA in approximately the same copy number over many somatic cell generations indicates the stability of these DNAs in the mitochondrion. Apparently the two molecules can be replicated independent of each other and the mitochondrial principal genome, as seen by the early replication amplification of the 1.9 kb

minicircle. These properties of the mitochondrial minicircle replicons could be useful in the production of recombinant DNA vectors. The minicircles could supply origins of replication for mitochondrial or nuclear vectors which are maintained autonomously.

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BIOGRAPHICAL SKETCH

Alan G. Smith was born in Lincoln, Nebraska, on January 16, 1956. He spent his formative years as the son of a gentleman farmer in Denton, Nebraska. He became an "excretaian" after graduation from Crete High School in 1974 and never again associated with this community. If he was not a BIG RED fan as a youth he was most certainly one after he received his Bachelor of Science degree in microbiology from the University of Nebraska in 1978. Graduate studies began at Iowa State University in the Department of Microbiology, where he received a Master of Science degree in 1981. His thesis research studied the attachment of cowpea mosaic virus to cowpea protoplasts and was supervised by Drs. J. H. Hill and D. P. Durand. Graduate study continued at the University of Florida, under the supervision of Drs. D. A. Pring and P. S. Chourey, where he completed the requirements for the degree of Doctor of Philosophy in 1985.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daryl R. Pring, Chairman
Professor of Plant Pathology

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Assistant Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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